



HAL
open science

Caractérisation de nouveaux substrats des sérine - thréonine protéine-kinases de mycobacterium tuberculosis

Marc Canova

► **To cite this version:**

Marc Canova. Caractérisation de nouveaux substrats des sérine - thréonine protéine-kinases de mycobacterium tuberculosis. Sciences agricoles. Université Claude Bernard - Lyon I, 2009. Français. NNT : 2009LYO10130 . tel-00599361

HAL Id: tel-00599361

<https://theses.hal.science/tel-00599361>

Submitted on 9 Jun 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



N° d'ordre : 130 – 2009



Année 2009

THESE DE L' UNIVERSITÉ DE LYON

délivrée par

L'UNIVERSITÉ CLAUDE BERNARD-LYON 1

Spécialité : Biochimie

DIPLÔME DE DOCTORAT

Soutenue publiquement le 16 septembre 2009

par

Marc CANOVA

**Caractérisation de nouveaux substrats des sérine/thréonine
protéine-kinases de *Mycobacterium tuberculosis***

Jury :
Dr. V. MOLLE
Pr. A. J. COZZONE
Dr. L. KREMER
Dr. D. MENGIN-LECREULX
Dr. R. BROSCHE

Directeur de thèse
Président
Examineur
Rapporteur
Rapporteur

*A Gilles, maintenant qu'il fait tout le temps nuit sur lui...
Je sais qu'il aurait préféré être là,
qu'il me voit,
veille sur moi,
et rigole
et rigole...*

Allez Neige, tombe comme avant, éclaire moi...

*A ma famille
Bien plus que celle du sang...*

*« On n'a jamais le temps, le temps nous a, il traîne
Comme un fleuve de plaine aux méandres moqueurs
Mais on y trouve un lit et des chants de sirènes
Et un songe accroché au pas du remorqueur
Jamais ce qui éteint, jamais ce qui dégoûte
Toujours, toujours, toujours, ce qui fait avancer
Il faut boire ses jours, un à un, goutte à goutte
Et ne trouver de l'or que pour le dépenser
Qu'on s'appelle Suzanne, Henri, Serge ou que sais-je
Quidam évanescant, anonyme, paumé
Il faut croire au soleil en adorant la neige
Et chercher le plus-que-parfait du verbe aimer
Il faut vivre d'amour, d'amitié, de défaites
Donner à perte d'âme, éclater de passion
Pour que l'on puisse écrire à la fin de la fête
Quelque chose a changé pendant que nous passions. »*

Claude Lemesle et Christian Piget

REMERCIEMENTS

Je tiens tout d'abord à sincèrement remercier l'ensemble des membres du jury, qui m'ont fait l'honneur de bien vouloir examiner ce travail. Je remercie Laurent Kræmer d'avoir accepté d'être examinateur, Dominique Mengin-Lecreulx et Roland Broseh d'avoir accepté d'être rapporteurs et Alain-Jean Cozzone de présider ce jury.

Merci au Pr. Cozzone de m'avoir accueilli à l'Institut de Biologie et Chimie des Protéines pour y effectuer ma thèse.

Je tiens à remercier, ma Directrice de Thèse, avec une ferveur particulière. Quatre ans passés avec toi pour « apprendre la Science ». Quatre ans de dur labeur avec mes hauts et mes bas, nos crises de rire au milieu du labo et les recadrages nécessaires du « Marco, ça va pas du tout là ». Mais, durant toutes ces années, tu ne m'as pas uniquement enseigné comment faire de beaux gels d'agarose. Impossible à présent d'ignorer que « le mieux est l'ennemi du bien » et que tu « aimes regarder Marco ». Grâce à toi, j'ai pu mûrir mon idée de la recherche et progresser tant au niveau technique que de l'écriture. Merci donc pour ta disponibilité, ton indulgence et tes encouragements. Merci aussi de m'avoir permis durant ces quatre années de développer d'autres aspects de moi en même temps que la thèse, l'enseignement (c'est quand qu'on trinque à mon ATER ???) et les stages BFA.

J'ai une tendresse particulière pour les Malin-Gérettés et pour nos longues discussions du midi. Merci à toi, Carole pour le courage que nous avons partagé durant ces quatre années, Merci à toi, Émeline, effroyable Garce et adorable Maman pour les sourires et la vie bienveillante que tu as su amener dans l'Institut en général et à notre table en particulier. Je te souhaite une longue vie dans la Science et toujours plus riche de jour en jour dans ta vie de super maman. Merci à toi, Stéphanie, pour tes sourires amusés à table lorsque que je racontais mes bêtises et pour ta bienveillante clémence envers mon humour plus que douteux. Harmonie, intelligence, raison ou sérénité, complices, connivence, autant de mots, pour exprimer tout ce que c'est...

Merci à Mick, Ben (Arthur!! Couillère!!) et Rachel, d'autres compagnons du midi pour nos nombreuses discussions scientifico-philosophiques. De la théorie de l'évolution aux relations amoureuses en passant par le sacré Graal grâce à une hirondelle d'Afrique, elle est où la poulette ?

Un très grand merci à Magali (Miss DVD) qui a su supporter toute mes vanes à deux centimes d'euro sans jamais m'envoyer bouler (hey, Mag, tu payes ton caf' ??). Merci à toi pour cette année de découvertes en tous genres et d'échanges culturels (accroche toi, tu vas y arriver), poursuit ta quête des vérités, scientifiques bien-sûr, mais surtout des autres vérités...

Un autre grand merci à Alex, qui maintenant est loin mais qui fût bien présent durant mes premières années. Merci à toi pour ta complicité dans le labo, pour les soirées (presque des nuits) au labo et les petits dèj' aux pains au chocolat. Merci aussi pour ta simplicité et ta gentillesse à mon égard. Merci encore pour les soirées « courses », les caissières gardent encore le souvenir ému de notre passage.

Merci à Agnès ainsi qu'à Karine pour tout le cœur qu'elles mettent à nous faciliter la vie, même quand ce n'est pas simple...

Les financements de toutes sortes mènent à bien des découvertes. Parfois scientifiques, parfois humaines, toujours, toujours, toujours enrichissantes...

Ainsi, je tiens à remercier Christelle qui fût rencontrée grâce à la Région (heureusement qu'elle était là cette bonne vieille Région...). Merci à toi pour tout ce que nous avons pu échanger durant nos dures années d'étude, à toutes nos discussions sur La Science et Les Hommes, à tout ce qui nous est cher et mille excuses pour les vanes sur les coconilles ou sur ton Miko chéri. Compagnone de Grimpe exemplaire, grâce à toi, je sais maintenant qu'on peut facilement enchaîner des 6a même après plusieurs mois d'absence.

Merci à Miko justement, pour avoir si souvent mis ses mains et son art au service de mon dos. HUUUUUUUU !! RRRrrrr !! Te quiero, cariño.

Merci à Lionel, mon autre compagnon de grimpe et mon belge préféré. Merci à toi pour ta sympathie et ton indulgence à mon égard, tant au niveau de la grimpe que de l'humour.

Merci à Thibault, ami de tous les bons moments de course à pied en resto top et grand complice de moqueries aux dépens de personnes ou de groupe de musique douteux!! Merci à toi pour toutes nos crises de rire...

Ma vie Lyonnaise fût avant tout faite de Science, mais pas uniquement, ce fût aussi le siège de merveilleuses rencontres et de découvertes de personnes inoubliables.

Mes remerciements vont donc en premier lieu à Ouelène, qui a su admirablement bien accompagner mes premières années lyonnaises (Hey Marco ! un p'tit rhum ?) et sans qui cette thèse ne serait pas tout à fait ce qu'elle est. Merci à Toi alors, adorable Blondinette, pour tous ces moments partagés, pour nos concours très classes à la fenêtre de mon appart, pour nos bols de sangria et pour les rencontres que tu as permises. Tu es maintenant loin mais tu manques à chaque instant. Bonne route à toi près de la mer et à ton amoureux. Souviens-toi que je ne suis qu'un fou et un homme pressé...

Mille mercis à mes amours Julien (mon docteur socio-économique) et Oriane (comment fais-tu pour encore rire à mes blagues??). Vous rencontrer fût une des meilleures choses qui me soit arrivées durant ces 4 ans de labeur. L'énumération de nos souvenirs communs serait trop longue mais merci pour tous ces moments en soirée ou autour d'un verre et de tout ce que vous m'avez apporté de bonne humeur, d'humour et de tendresse.

Merci à Etienne (allo Grenoble ?)! Homme de droit blanc sur un échiquier noir. De la même façon une dizaine de pages ne suffirait pas à narrer par le menu toutes les niaiseries et les délires dont nous fûmes coupables. Tu seras pour toujours mon spécialiste préféré des ogres (attention, interro des paroles très bientôt...). J'te l'raconte comme ça, si j'avais été jolie fille...

Merci à toutes les belles personnes que j'ai rencontrées grâce aux formations BAF.

Je pense bien entendu à Cédric et Mylène, qui ont su accompagner ma vie de thésard avec tant d'attention. Merci à vous mes amis pour nos longues discussions, pour les coups de cœur et de pieds, pour votre aide et votre soutien. Merci aussi pour tout l'amour et la tendresse dont vous faites preuve à mon endroit. Même tous les Mots d'Amour ne suffiraient pas à vous rappeler tout cela, en vous serrant dans mes bras...

Merci à Amélie (en avant les histoires !!) qui m'a prouvé, entre autres, scientifiquement que les antibiotiques et la fatigue, ça fait pas rire pour expliquer un projet de recherche et à Cha (ma « p » préf de préf) et ses ailes de papillons bleus. Merci à vous deux pour tous ces moments partagés en w-e NF ou durant les stages (Armageddon !!).

Un grand merci aussi à toute l'équipe des Pirates, Valérie (la cheffe des pirates), Marine, Anne-laure et les moussaillons Anaëlle et Antoine pour ces longues semaines remplies de joies et d'aventures en tous genres.

Enfin, je souhaiterais bien sûr remercier ma famille.

Merci à mes parents de m'avoir toujours donné les moyens de trouver un sens et un but à ma vie, de m'avoir toujours soutenu et encouragé dans mes moments de doute et d'avoir mis toute la bonne volonté du monde à tenter de comprendre le sens profond de mes travaux de recherche.

Merci à mes deux adorables frangines. A Teutzu qui a donné naissance à un merveilleux loulou, tout beau, tout gros, tout rose nommé Paul et à mon Boubou d'Amour qui a su me livrer la fameuse recette de ces patés à la carbo. Merci à vous de supporter mon esprit sarcastique grandissant...

Merci à mon adorable Laura, rencontrée grâce à la Science (comme quoi...) qui a su entrer dans ma vie et y faire sa place. Merci pour les sourires du matin qui illuminent des journées entières, pour ta patience quand je perds mes billes et pour la vie que nous partageons au quotidien.

Merci à ma Belle Nadia, petite gazelle du désert qui dans le secret de son magnifique couple a pris le temps de faire un enfant avec l'homme de sa vie. Tu vois ma Belle, finalement, les montées quizz ont fini par porter leur fruit...

Merci à Gaëlle pour nos virées au Me Do/réconciliation post-prise de tête chaque fois que tu redescends de ton Nord-Est froid et pluvieux. Depuis le DEUG à Grenoble, que de chemin pour toi et moi... Merci de ton éternel soutien !!

Merci aussi à Vincent (Mon gros loup) pour l'Amour inconditionnel que l'on se voue mutuellement depuis le magistère (oui je sais, toi tu n'aimes personne...). Merci à toi pour toutes ces années passées à tes côtés, pour toutes les découvertes que nous avons faites ensemble et pour les tas et tas et tas de délires partagés. Tu vois finalement les armes en mousse, ça aide quand même, même si on tape fort...

Merci à Simon (le troll des bois), Laurette (la magicienne des grottes) et Matthew (The ours), autres compagnons de route depuis le magistère qui ont su accompagner mes pas de leur amitié bienveillante, chaleureuse et tendre. Il me semble qu'il nous reste encore quelques sommets à gravir et que certains ruisseaux n'attendent que nous...

Merci aussi à Aurélie (le petit korrigan des prés fleuris) pour ton soutien durant ma rédaction et pour toutes nos joies passées et à venir.

Merci à Sandra pour ses passages surprises durant ma vie lyonnaise et dans ma vie tout court. J'espère que la vie fera se croiser nos chemins encore de nombreuses fois...

Merci à Philippe, mon prof de guitare préféré, j'espère être un enfant qui réussira toujours.

RÉSUMÉ

Les microorganismes possèdent un ensemble de mécanismes leur permettant de percevoir la nature et les modifications du milieu dans lequel ils évoluent, et d'adapter en conséquence leur métabolisme et leur physiologie. Chez les bactéries, il existe un système de régulation via la phosphorylation semblable à celui retrouvé classiquement chez les eucaryotes, qui permet la modification des protéines aux dépens de l'ATP sur un nombre restreint d'acides aminés : la sérine, la thréonine et la tyrosine. Le séquençage intégral du génome de *Mycobacterium tuberculosis* a permis de mettre en évidence l'existence de onze Sérine/Thréonine Protéine-Kinases (STPKs) chez cette bactérie. Bien que la quasi-totalité des STPKs aient été biochimiquement caractérisées, très peu de substrats endogènes ont pu être identifiés. Par conséquent, le rôle physiologique de ces couples kinase/substrat reste à élucider.

Tout d'abord, les études réalisées au cours de ce travail ont concerné la caractérisation biochimique de la protéine-kinase PknL, ainsi que l'identification de ses substrats potentiels, et notamment la protéine Rv2175c. En effet, l'analyse de l'environnement génétique du gène *pknL* de la kinase a révélé la présence du gène adjacent *rv2175c*, pouvant ainsi représenter un substrat éventuel de PknL. Les différentes approches mises en œuvre ont permis d'identifier cinq sites de phosphorylation sur PknL, et de mettre en évidence le caractère essentiel des résidus K48, T173 et T175 dans les mécanismes d'autophosphorylation de PknL et de phosphorylation de Rv2175c, confirmant ainsi Rv2175c comme substrat spécifique de PknL. Par ailleurs, la caractérisation par RMN de la structure de Rv2175c a permis de déterminer la fonction de cette protéine. Rv2175c possède toutes les caractéristiques structurales d'une protéine capable de fixer l'ADN. Des études fonctionnelles ont permis de confirmer la capacité de Rv2175c de fixer l'ADN et ont mis en évidence le mécanisme de régulation *via* phosphorylation régissant son activité de fixation. Ensuite, nous avons mis en évidence la phosphorylation des protéines chaperonnes mycobactériennes et, plus particulièrement, caractérisé GroEL1. Nous avons démontré que GroEL1 était phosphorylée par PknF, et identifié les résidus T25 et T54 comme étant les sites de phosphorylation de GroEL1.

L'ensemble de cette étude nous a donc permis de caractériser de nouveaux substrats de phosphorylation chez *M. tuberculosis*, de mieux appréhender les interactions kinase/substrat et d'impliquer la phosphorylation dans la régulation de l'activité de ces substrats.

Mots-clé : *Mycobacterium tuberculosis*, phosphorylation, sérine/thréonine protéine-kinase

SUMMARY

Mycobacterium tuberculosis (*M. tb*) has a complex life style comprising different environments and developmental stages. The success of *M. tb* results from its remarkable capacity to survive within the infected host, where it can persist for several decades, which is linked to the presence of its unusual cell wall. However, little is known regarding its capacity to modulate and adapt expression of cell wall components in response to environmental changes.

Signal sensing leading to cellular responses must be tightly regulated to allow survival under variable conditions. Prokaryotes generally control their signal transduction processes through two-component systems. However, the genome of *M. tb* revealed a large family of eukaryotic-like Ser/Thr Protein-Kinases (STPKs). It is becoming clear that in *M. tb* many of these kinases are involved in the regulation of metabolic processes, transport of metabolites, cell division or virulence, and therefore signalling through Ser/Thr phosphorylation has emerged as a critical regulatory mechanism in pathogenic mycobacteria. Still, our understanding of mycobacterial kinase biology has been seriously hampered by the failure to identify relevant kinase substrates. Consequently, identification and investigation of the biochemistry and physiological role of STPK substrates should lead to a better understanding of the complex signalling networks in *M. tb*.

Analysis of the genome sequence of *Mycobacterium tuberculosis* predicted the presence of eleven Serine / Threonine Protein-Kinases (STPKs). Although most kinases have been investigated for their physiological roles, little information is available regarding how STPK-dependent phosphorylation regulates the activity of kinase substrates. As a result, the physiological role of these kinase / substrate couples remains to be clarified.

During the course of this work, we first characterized a substrate/kinase pair, PknL/Rv2175c. The *pknL* gene is associated with the ~30 kb *dcw* (*d*ivision *c*ell *w*all) gene cluster which encompasses several genes involved in cell wall synthesis and cell division, raising the possibility that PknL might participate in the regulation of this gene cluster. Moreover, *pknL* (*rv2176*) is adjacent to *rv2175c*, a gene encoding a putative DNA-binding transcriptional regulator. We demonstrated that PknL can recruit and phosphorylate Rv2175c and that phosphorylation of Rv2175c was dependent on a specific phosphorylated residue located within the activation loop of PknL. However, although Rv2175c harbours a DNA-binding domain carrying a helix-turn-helix (HTH) motif, it shares only weak similarity to transcriptional regulatory proteins. Therefore, to provide further evidence for the function of Rv2175c, we have solved the soluble NMR structure of Rv2175c. This unambiguously confirmed that Rv2175c is a DNA-binding protein with a HTH domain. In addition, we

confirmed by gel shift mobility assays that Rv2175c was indeed able to bind DNA. More importantly, we identified Thr9 as the unique phosphorylation site in Rv2175c, and demonstrated that phosphorylation of Rv2175c strongly altered its DNA-binding activity.

In addition, although mycobacterial GroEL1 proteins have been extensively studied, no data were available with respect to their potential post-translational modifications. We reported here, for the first time, phosphorylation of the *M. tuberculosis* GroEL1 chaperone. We demonstrated that *M. tb* GroEL1 is phosphorylated by PknF at two positions, Thr25 and Thr54. Unexpectedly, *Mycobacterium smegmatis* GroEL1 is not a substrate of its cognate PknF. This study showed that the phosphorylation profile of conserved proteins is species-dependent and provides insights that may explain the numerous biological functions of these important proteins.

Finally, we constructed an improved vector, pETPhos, to synthesize proteins harboring a N-terminal His-tag fusion, which can be efficiently removed using the TEV protease. One major advantage of pETPhos relies on the lack of Ser and Thr residues in the fusion tag, representing potential non-specific phosphorylation sites. We demonstrated that pETPhos represents a valuable tool for characterization of the phosphoacceptors in bacterial STPKs, and presumably also in Tyr protein kinases, as well as in their substrates.

LISTE DES ABRÉVIATIONS

- ABC : ATP Binding Cassette
- AceK : Isocitrate déshydrogénase kinase/phosphatase
- ADN : Acide désoxyribonucléique
- ADP : Adénosine diphosphate
- AMPC : Adénosine monophosphate cyclique
- ARN : Acide ribonucléique
- ATP : Adénosine triphosphate
- BY-kinase : Bacterial Tyrosine Kinase
- DTT : dithiotréitol
- FAS : Fatty acid synthase
- FHA : Forkhead associated
- GSNO : S-nitroglutathion
- HTH : Hélice-Tour-Hélice
- IDH : Isocitrate déshydrogénase
- LAM : lipoarabinomannane
- MAP-kinases : Mitogen-activated protein-kinases
- MBP : Myelin Basic Protein
- MDR-TB : Tuberculose multirésistante
- Mg²⁺ : ion magnésium
- MSF : Médecins sans frontière
- NADPH : Nicotinamide adénine dinucléotide phosphate
- OMS : Organisation Mondiale de la Santé
- PASTA : Penicillin-binding protein And Serine/Threonine kinase Associated
- Pi: phosphate inorganique
- RMN : Résonance Magnétique Nucléaire
- SARP : *Streptomyces coelicolor* Antibiotic Regulatory Proteins
- SIDA : Syndrome de l'Immunodéficience Acquise
- SNP : nitroprussiate de sodium
- STPK(s) : Sérine/Thréonine Protéine-kinase (s)
- TRP : tetratricopeptide

VIH : Virus de l'Immunodéficience Humaine

WNL : With No K (lysine)

XDR-TB : Tuberculose ultrarésistante

TABLES DES MATIÈRES

<i>Introduction</i>	1
<i>Chapitre 1</i>	3
<i>Exposé bibliographique</i>	3
1.1 LA PHOSPHORYLATION DES PROTEINES	4
1.1.1 Les systèmes à deux composants His/Asp	5
1.1.2 Les protéine-kinases de type Ser/Thr/Tyr	6
1.1.2.1 Les Ser/Thr/Tyr protéine-kinases de type Hanks	6
1.1.2.2 Les autres Ser/Thr/Tyr protéine-kinases	11
1.2 MYCOBACTERIUM TUBERCULOSIS	15
1.2.1 Dépistage, traitement et prévention de la tuberculose	16
1.2.1.1 Dépistage	16
1.2.1.2 Le traitement antibiotique	16
1.2.1.3 La prévention	18
1.2.2 <i>M. tuberculosis</i> , l'agent étiologique de la tuberculose humaine	18
1.2.2.1 Le genre <i>Mycobacterium</i>	18
1.2.2.2 Le cycle infectieux	19
1.3 LES PROTÉINE-KINASES DE MYCOBACTERIUM TUBERCULOSIS	20
1.3.1 Groupe 1 : PknA/B/L	20
1.3.1.1 PknA	20
1.3.1.2 PknB	22
1.3.1.3 PknL	24
1.3.2 Groupe 2 : PknD/E/H	25
1.3.2.1 PknD	25
1.3.2.2 PknE	26
1.3.2.3 PknH	27
1.3.3 Groupe 3 : PknF/PknI/PknJ	29
1.3.3.1 PknF	29
1.3.3.2 PknI	30
1.3.3.3 PknJ	31
1.3.4 Groupe 4 : PknK et PknG	31
1.3.4.1 PknK	31
1.3.4.2 PknG	32

Chapitre 2	34
Résultats	34
<u>Article I</u> : The <i>Mycobacterium tuberculosis</i> serine/threonine kinase PknL phosphorylates Rv2175c: mass spectrometric profiling of the activation loop phosphorylation sites and their role in the recruitment of Rv2175c.	37
<u>Article II</u> : The <i>Mycobacterium tuberculosis</i> Ser/Thr kinase substrate Rv2175c is a DNA-binding protein regulated by phosphorylation.	52
<u>Article III</u> : pETPhos: a customized expression vector designed for further characterization of Ser/Thr/Tyr protein kinases and their substrates.	74
<u>Article IV</u> : The <i>Mycobacterium tuberculosis</i> GroEL1 chaperone is a substrate of Ser/Thr Protein Kinases.	81
<u>Article annexe I</u> : From the characterization of the four serine/threonine protein kinases (PknA/B/G/L) of <i>Corynebacterium glutamicum</i> toward the role of PknA and PknB in cell division.	91
<u>Article annexe II</u> : The MurC ligase essential for peptidoglycan biosynthesis is regulated by the serine/threonine protein kinase PknA in <i>Corynebacterium glutamicum</i>	91
 Chapitre 3	 118
Discussion et Conclusion	118
 Bibliographie	 126

INTRODUCTION

La cellule doit s'adapter aux conditions environnementales auxquelles elle est exposée afin de se reproduire lorsque ces conditions sont favorables, ou d'assurer le maintien de fonctions cellulaires essentielles à sa survie lorsque ces conditions deviennent critiques. Cette adaptation est médiée par des voies de signalisation qui permettent, au niveau moléculaire, de transmettre un signal ou stimulus de l'extérieur vers l'intérieur de la cellule, celle-ci pouvant alors émettre une réponse appropriée et ajuster ses processus cellulaires aux variations environnementales. La transduction du signal implique des séquences ordonnées de réactions biochimiques catalysées par des enzymes aboutissant à la modification des caractéristiques telles que la charge, l'hydrophobicité, la conformation et/ou la stabilité de la protéine concernée. Ainsi, la modification chimique d'un acide aminé permet, au niveau de la protéine, de réguler son activité, sa localisation, son transport, ou ses interactions avec d'autres protéines.

La phosphorylation des protéines est une modification post-traductionnelle covalente que subissent certaines protéines, catalysée par des enzymes spécifiques : les protéine-kinases. Ce phénomène est réversible et les phosphoprotéines peuvent, en retour, être déphosphorylées par des phosphoprotéine-phosphatases. Ainsi, les réactions de phosphorylation/déphosphorylation induisent une modification de l'activité ou de la structure des protéines et permettent la régulation de différentes voies métaboliques.

Alors qu'il était largement admis que les systèmes de régulation bactériens étaient principalement contrôlés par un mécanisme de phosphorylation faisant intervenir les systèmes « à deux composants », la mise en évidence de protéines homologues aux kinases et phosphatases eucaryotes a permis d'ouvrir un champ d'investigation totalement nouveau concernant le rôle des Sérine/Thréonine Protéine-Kinases (STPKs) bactériennes dans les processus de régulation cellulaire. Chez la bactérie *Mycobacterium tuberculosis*, la transduction du signal s'effectue à la fois grâce à des systèmes à deux composants et grâce à la modification des protéines via les STPKs. Les travaux réalisés précédemment au laboratoire ont conduit, d'une part, à la caractérisation de différentes STPKs de *Mycobacterium tuberculosis* et, d'autre part, à l'identification de différents substrats des STPKs mycobactériennes. Ces travaux ont aussi permis de mettre en évidence différents mécanismes d'interaction kinase/substrat, et donc de mettre à jour leur implication au niveau de cascades de phosphorylation présentes dans différentes voies de régulation. Par exemple, le rôle et l'importance de domaines particuliers appelés FHA (ForkHead Associated) ont été démontrés dans les mécanismes de recrutement et de phosphorylation des protéines EmbR et Rv1747 par les kinases PknH et PknF, respectivement. De plus, d'autres études ont également montré que les enzymes

KasA et KasB correspondaient à des substrats spécifiques des STPKs. Ces enzymes sont impliquées dans les voies de biosynthèse des acides mycoliques, composants de la paroi de *M. tuberculosis*.

Au sein du laboratoire, mes travaux se sont inscrits dans cette optique de caractérisation biochimique des STPKs de *M. tuberculosis*, ainsi que dans la recherche et l'identification de substrats de phosphorylation. Mon projet a porté, tout d'abord, sur la caractérisation du couple protéine-kinase/substrat, PknL/ Rv2175c. Dans la continuité de ce projet, j'ai cherché à analyser de manière plus détaillée la fonction de la protéine Rv2175c et l'incidence de la phosphorylation sur cette fonction. Mon étude s'est ensuite étendue à la recherche d'autres substrats de phosphorylation. Mon activité s'est alors concentrée sur la mise en évidence de la phosphorylation de GroEL1, protéine chaperonne de *M. tuberculosis*, par PknF et sur l'identification des sites de phosphorylation de GroEL1.

Chapitre **1**

Chapitre 1 : Exposé bibliographique

La première partie de cet exposé bibliographique décrira les principales connaissances scientifiques concernant la phosphorylation des protéines chez les bactéries.

La deuxième partie sera consacrée à la description du modèle d'étude, *Mycobacterium tuberculosis*.

Finalement, la troisième partie de ce chapitre exposera l'ensemble des données connues, à ce jour, sur les onze STPKs de *M. tuberculosis*.

1.1 LA PHOSPHORYLATION DES PROTÉINES

Chez les eucaryotes, le rôle régulateur de la phosphorylation a pu être mis en évidence pour la première fois par Krebs et Fischer, qui ont démontré la modulation de l'activité de la glycogène phosphorylase du muscle squelettique de lapin selon son état de phosphorylation (Krebs & Fischer, 1956). La phosphorylation de protéines catalysées par des protéine-kinases n'a été observée chez les procaryotes qu'à la fin des années 70. En effet, en 1979, Garnak et Reeves ont démontré chez *Escherichia coli* que l'activité catalytique de l'isocitrate déshydrogénase était régulée par phosphorylation sur un résidu sérine. Les études pionnières menées par A. J. Cozzone et son équipe ont indiqué ensuite l'existence de 128 protéines phosphorylées chez *E. coli* (Cortay *et al.*, 1986). L'identification des protéines phosphorylées s'est accrue récemment notamment grâce au développement d'approches globales : l'étude des phosphoprotéomes. De telles études ont été conduites chez *Corynebacterium glutamicum* (Bendt *et al.*, 2003), *Bacillus subtilis* (Levine *et al.*, 2006 ; Macek *et al.*, 2007), *E. coli* (Macek *et al.*, 2008), et *Lactococcus lactis* (Soufi *et al.*, 2008).

D'un point de vue chimique, la phosphorylation correspond au transfert d'un groupement phosphate d'un donneur à un accepteur. De manière générale, le donneur est un nucléoside triphosphate, le plus souvent l'ATP, et l'accepteur est la chaîne latérale d'un acide aminé. Cette réaction réversible est catalysée par des enzymes spécifiques, les protéine-kinases dans le sens protéine-phosphoprotéine et les phosphoprotéine-phosphatases dans le sens phosphoprotéine-protéine (Figure n°1). La liaison du groupement phosphate à l'accepteur diffère selon les groupements de celui-ci. En principe, il en existe quatre types possibles : liaison phospho-ester avec les résidus séryles, thréonyles et tyrosyles; liaison phospho-amide avec la lysine, l'arginine et l'histidine; liaison de type anhydre avec l'aspartate et le glutamate, et liaison phospho-thioester avec

la cystéine (Duclos *et al.*, 1991). La phosphorylation sur les résidus cystéine est rare. Seules quelques protéines phosphorylées sur des résidus cystéyles ont été découvertes chez *E. coli* : la thiorédoxine et les transporteurs EIIMtl et IIBCGIC (Pigiet & Conley, 1978 ; Pas & Robillard, 1988 ; Meins *et al.*, 1993). La liaison phosphate-cystéine apparaît aussi transitoirement comme intermédiaire de réaction dans les déphosphorylations effectuées par certaines classes de phosphatases, comme Yop51, une tyrosine phosphatase de *Yersinia pestis* (Stuckey *et al.*, 1994).

Dans le chapitre suivant, nous détaillerons les connaissances actuelles sur les systèmes à deux composants His/Asp, les mieux étudiés chez les bactéries, ainsi que les systèmes de type Ser/Thr/Tyr qui font plus précisément l'objet de cette thèse.

1.1.1 Les systèmes à deux composants His/Asp

Le système de phosphorylation le mieux étudié chez les bactéries est le système dit « à deux composants ». Il est à la base du transfert de l'information qui conditionne le comportement de la cellule en réponse à un changement environnemental. Dans ce système, deux éléments conservés ont été identifiés sur la base des alignements de séquences en aminoacides (Nixon *et al.*, 1986). Ils se retrouvent habituellement sur une protéine-kinase senseur et sur un régulateur de réponse. En réponse à un stimulus environnemental, la protéine-kinase senseur (ou histidine protéine-kinase) s'autophosphoryle aux dépens de l'ATP sur un résidu histidyle présent dans son domaine C-terminal conservé. Le groupement phosphate est ensuite transféré sur un résidu aspartyle présent dans le domaine N-terminal, lui aussi conservé, du régulateur de réponse (ou protéine régulatrice) (Figure n°2). Le régulateur de réponse ainsi phosphorylé va se fixer aux régions promotrices de gènes cibles *via* un domaine Hélice-Tour-Hélice (HTH) et induire leur expression (Hoch, 2000). Dans quelques cas, il a été montré que l'activité His protéine-kinase de la protéine senseur et l'activité de la protéine effectrice pouvaient être portées par la même molécule. Dans d'autres cas, au contraire, trois protéines distinctes catalysent respectivement les différentes activités, sensorielle, protéine-kinase et effectrice (Saier *et al.*, 1990).

La variabilité des domaines additionnels non impliqués dans le transfert du groupement phosphate permet l'intervention de ce système de transduction du signal dans la régulation d'un grand nombre de fonctions cellulaires. Les systèmes à deux composants bactériens, véritables "senseur" de l'environnement, sont impliqués dans l'utilisation des métabolites (azote, carbone ou phosphate). Ils affectent la différenciation, la compétence cellulaire, la sporulation et d'autres fonctions comme le chimiotactisme, la synthèse ou la résistance aux antibiotiques. Les systèmes à deux composants sont également importants pour l'adaptation bactérienne (à un choc osmotique,

stress, anaérobiose et carence alimentaire), et pour la virulence, capable de détecter les changements de conditions environnementales et moduler en retour l'expression de gènes spécifiques (Cozzone, 1998a).

Ces systèmes sont en général très abondants chez les bactéries, ils sont présents aussi bien chez les espèces à Gram-positif que chez celles à Gram-négatif. Mais leur nombre varie selon les microorganismes : aucun système de ce type n'existe chez *Mycoplasma genitalium* alors qu'il en existe 80 chez *Synechocystis* sp. strain PCC 6803 (Mizuno, 1998).

Il est intéressant de noter que, récemment, ce système de phosphorylation a été également détecté chez des organismes autres que des bactéries comme chez la plante *Arabidopsis thaliana* où il joue un rôle dans la régulation de l'utilisation de l'éthylène (Chang & Meyerowitz, 1995), et chez la levure *Saccharomyces cerevisiae* où il intervient dans la régulation d'une cascade de MAP kinases impliquées dans l'osmorégulation (Ota & Varshavsky, 1993). Ces exemples laissent penser qu'un mode de transmission de certains signaux similaire à celui des bactéries pourrait être présent chez les organismes eucaryotes.

1.1.2 Les protéine-kinases de type Ser/Thr/Tyr

Les Ser/Thr/Tyr protéine-kinases identifiées à ce jour ont été classées suivant plusieurs critères complémentaires. Ainsi, l'analyse des motifs structuraux conservés chez les protéine-kinases définit une large famille de kinases, les kinases de type Hanks (Hanks *et al.*, 1988), alors que les kinases n'appartenant pas à cette famille ont été classées selon leur mécanisme d'action et/ou leur spécificité envers l'acide aminé phosphorylé. Dans les chapitres suivants, nous nous intéresserons essentiellement aux familles de Ser/Thr/Tyr kinases présentes chez les bactéries.

1.1.2.1 Les Ser/Thr/Tyr protéine-kinases de type Hanks

Les Ser/Thr/Tyr protéine-kinases identifiées comme kinases de type eucaryote appartiennent à la famille des protéine-kinases de type Hanks. Cette famille définie par homologie de séquence est divisée en deux groupes: les Ser/Thr protéine-kinases et les Tyr protéine-kinases (Hanks *et al.*, 1988 ; Hanks & Hunter, 1995). De nombreuses protéines ont pu être identifiées comme protéine-kinases appartenant à la famille de Hanks grâce au développement de techniques comme la PCR et le séquençage. En effet, les protéines de cette famille montrent au niveau de leur domaine catalytique des motifs conservés permettant une identification relativement aisée de nouvelles protéines par alignement de séquence avec des membres connus (Figure n°3). Les programmes

systématiques de séquençage des génomes ont permis la détection de nombreuses protéine-kinases putatives chez les eucaryotes. Par exemple, chez *Caenorhabditis elegans*, sur 19 099 protéines potentielles, 585 protéines ont été prédites comme protéines appartenant à la famille de Hanks, dont 493 Ser/Thr protéine-kinases et 92 Tyr protéine-kinases (Plowman *et al.*, 1999). Le séquençage du génome humain a permis de détecter plus de 500 Ser/Thr/Tyr protéine-kinases putatives (Manning *et al.*, 2002).

Chez les procaryotes, certaines protéines-kinases de la famille de Hanks ont été identifiées et caractérisées par des approches moléculaires et biochimiques, comme le montrent Munoz-Dorado et collaborateurs, qui ont ainsi mis en évidence la première-kinase bactérienne de type eucaryote, Pkn1, chez *Myxococcus xanthus* (Munoz-Dorado *et al.*, 1991). Ils ont, pour ce faire, utilisé des oligonucléotides conçus à partir de régions hautement conservées de Ser/Thr protéine-kinases eucaryotes et ont également démontré l'activité d'autophosphorylation de Pkn1 sur des résidus séryles et thréonyles *in vitro*. Mais, de même que pour les eucaryotes, c'est la généralisation du séquençage des génomes bactériens qui a permis la découverte de nouveaux membres de cette famille (Shi *et al.*, 1998).

D'un point de vue structural, les protéine-kinases de la famille de Hanks sont généralement composées d'un domaine catalytique et d'une sous-unité régulatrice. La séquence primaire du domaine catalytique est très conservée. Ce domaine est constitué de 250 à 300 acides aminés. L'alignement de la séquence primaire des domaines catalytiques des kinases de type Hanks a permis de visualiser l'alternance de 12 sous-domaines conservés avec des régions de moindre conservation, des lacunes ou des insertions (Hanks *et al.*, 1988 ; Taylor, 1989) (Figure n°3). Les régions non conservées constituent le plus souvent des boucles dont le repliement permet l'agencement tri-dimensionnel des régions conservées. Les sous-domaines conservés ont été numérotés de I à XI, le sous-domaine VI étant divisé en deux régions VIA et VIB. Les domaines catalytiques des protéine-kinases peuvent être trouvés sous deux états conformationnels, appelés conformations ouverte et fermée (Cox *et al.*, 1994). De manière générale, la conformation fermée correspond à l'état actif de ces enzymes.

La protéine-kinase AMPc-dépendante PKA a été la deuxième kinase purifiée chez les eucaryotes (Walsh *et al.*, 1968) et son domaine catalytique PKA-C α , cristallisé en conformation fermée, est utilisé comme protéine-kinase de référence (Knighton *et al.*, 1991a, 1991b ; Walsh *et al.*, 1992). Le domaine catalytique de PKA-C α est organisé de manière bilobale hautement ordonnée, où chaque sous-domaine représente une unité précise. Ces sous-domaines contiennent tous des résidus très peu variables, dont 12 quasiment invariants (conservés dans plus de 95 % des séquences). Le lobe comprenant la partie N-terminale de la protéine, appelé petit lobe, est organisé en feuillets β anti-parallèles. Il comprend les sous-domaines I à IV et est impliqué dans la fixation et

l'orientation du complexe ATP/Mg²⁺. Relié au petit lobe par le sous domaine V, le grand lobe comprend la partie C-terminale ainsi que les domaines VIA à XI. Il est en partie responsable de la liaison du peptide substrat et de l'initiation du transfert de phosphate.

– Dans le sous-domaine I se trouvent les résidus essentiels G50 et G52. Ces deux glycines font partie d'un motif GxGxxG, situé dans un coude serré qui relie deux feuillets β anti-parallèles, permettant de couvrir les phosphates non transférables de l'ATP. Il apparaît donc que ces glycines sont importantes pour la fixation et le relâchement de l'ATP (Hemmer *et al.*, 1997).

– Le sous-domaine II et le sous-domaine III contiennent chacun un résidu invariable, le résidu K72 et le résidu E91, respectivement. K72 (du motif AxK) intervient dans l'orientation et la liaison de l'ATP en interagissant avec les groupements phosphate α et β . Par ailleurs, K72 interagit avec E91 par le biais d'un pont salin, facilitant les interactions de la lysine et des groupements phosphate de l'ATP. De plus, cette lysine semble être impliquée dans le transfert du groupement phosphate (Carrera *et al.*, 1993). Il est important de noter que la modification de cette lysine engendre la perte de l'activité kinase chez de nombreuses protéine-kinases de type Hanks (Zoller *et al.*, 1981 ; Wick *et al.*, 2002). Au début des années 2000, un nouveau groupe de protéine-kinases actives sans le résidu essentiel lysyle a été mis en évidence et appelé les WNK (With No lysine (K)) (Xu *et al.*, 2000). Des études ultérieures ont cependant montré que les WNK possèdent une lysine catalytique située sur le feuillet β_2 du sous-domaine I, au lieu du feuillet β_3 du sous-domaine II (Xu *et al.*, 2002). À ce jour, aucune protéine-kinase de ce type n'a été découverte chez les bactéries.

– Le sous-domaine IV, de par son absence de résidu essentiel, ne semble pas être directement impliqué dans la catalyse ou la reconnaissance du substrat.

– Le sous-domaine V, qui lie le petit lobe au grand lobe, définit le site catalytique de l'enzyme (Hanks & Hunter, 1995).

– Le motif conservé du sous-domaine VIB, DxKxxN (D166, K168 et N171 chez PKA-C α), forme la boucle catalytique. Celle-ci est le pivot central qui communique avec les différentes parties de la molécule, et permet l'orientation du peptide afin de favoriser la catalyse. Le résidu aspartyle et le résidu lysyle ont un rôle direct dans le transfert du phosphate. Le premier joue le rôle d'accepteur de proton du substrat et le second neutralise les charges négatives du phosphate γ . Le résidu asparagyle a pour rôle d'aider à la stabilisation de la boucle catalytique (Hanks & Quinn, 1991).

- Le triplet DFG (résidus 184 à 186) du sous-domaine VII permet l'orientation du phosphate γ lors du transfert. Deux ions métalliques sont liés à l'ATP. Le premier ion chélate les phosphates β et γ de l'ATP ainsi que le résidu invariable D184. Le second ion coordonne les phosphates α et γ de l'ATP et de la chaîne latérale du résidu asparagyle 171 (Bossemeyer, 1995).
- Le sous-domaine VIII, contenant le motif APE (résidus 206 à 208 de PKA-C α) semble jouer un rôle majeur dans la reconnaissance des peptides substrats. De plus, le résidu glutamyle interagit avec le résidu arginyle 280 du sous-domaine XI, stabilisant le grand lobe.
- L'aspartate 220 du sous-domaine IX renforce la position de la boucle catalytique en formant des liaisons hydrogène avec d'autres acides aminés (R165 et Y164) (Bossemeyer, 1995).
- Le sous-domaine X est le moins bien conservé et sa fonction reste encore obscure.
- Enfin, le dernier sous-domaine XI se situe sur la partie C-terminale du domaine catalytique et contient un résidu invariable, l'arginine 280.

De nombreuses protéine-kinases sont activées par phosphorylation de résidus localisés au centre du domaine kinase, leur nombre et leur emplacement variant en fonction de l'enzyme. Ce segment, également appelé boucle d'activation, est défini comme la région comprise entre les sous-domaines VII (DFG) et VIII (APE), et correspond aux résidus 184-208 chez la PKA-C α (Taylor & Radzio-Andzelm, 1994). Par exemple, l'activité de la PKA-C α est maximale quand la thréonine 197 de la boucle d'activation est phosphorylée (Steinberg *et al.*, 1993). Dans la majorité des cas, la boucle d'activation sous forme déphosphorylée adopte une conformation à l'intérieur du site actif de l'enzyme, bloquant ainsi l'entrée et la liaison du peptide substrat. Après sa phosphorylation, la boucle d'activation se positionne en retrait du centre catalytique. Ces modifications de conformation permettent l'entrée de l'ATP dans le site actif ainsi que la liaison et l'orientation correcte du peptide substrat. De plus, la portion N-terminale de la boucle d'activation est positionnée de manière à placer, à l'intérieur du site actif, le motif conservé DFG du sous-domaine VII, nécessaire à l'orientation du phosphate γ lors du transfert (Johnson *et al.*, 1996 ; Huse & Kuriyan, 2002).

La similarité topologique des domaines catalytiques des protéine-kinases bactériennes de type Hanks avec ceux des eucaryotes pose le problème de leur origine. Sur la base d'une analyse phylogénique, il a été initialement proposé que les protéine-kinases bactériennes de type Hanks résulteraient d'un transfert horizontal précoce entre les eucaryotes et les procaryotes (Leonard *et al.*,

1998). Cette hypothèse s'appuyait, à l'époque, sur l'absence de protéine-kinases de ce type identifiée chez les archae. En fait, bien que non clairement établie, des analyses suggèrent fortement la présence de ce type de kinase chez les archae (Bidle, 2003). L'étude conduite chez la cyanobactérie *Synechocystis* sp. strain PCC 6803 par Han et Zhang en 2001 indique plutôt une origine commune des kinases bactériennes et eucaryotes, avant la divergence eucaryote/procaryote dans l'évolution. En effet, le contenu en bases GC des gènes codant les Ser/Thr kinases de *Synechocystis* et l'usage des codons sont similaires à la moyenne des autres gènes du génome, soutenant ainsi l'origine bactérienne de ces gènes. Le fait que ces protéine-kinases soient retrouvées dans tous les génomes bactériens séquencés va à l'encontre de l'idée d'un transfert horizontal des eucaryotes aux procaryotes (Han & Zhang, 2001). Actuellement, l'origine précise des protéine-kinases bactériennes n'est toujours pas clairement établie (Kannan *et al.*, 2007). Différentes sous-unités régulatrices peuvent être associées aux domaines kinases et déterminent très probablement le rôle fonctionnel précis des protéine-kinases de type Hanks chez les bactéries.

D'un point de vue distribution et fonction de ces kinases, Krupa et Srinivasan ont décrit, sur la base d'une analyse comparative de séquences, divers domaines rencontrés chez les protéines-kinases bactériennes (Krupa & Srinivasan, 2005). On trouve notamment le domaine PASTA (penicillin-binding protein and serine/threonine kinase associated domain) conservé chez les bactéries à Gram-positif (Yeats *et al.*, 2002). Ce domaine C-terminal a été identifié pour la première fois dans la protéine PBP2X chez *Streptococcus pneumoniae* où il est répété deux fois, et sa structure a été déterminée par cristallographie aux rayons X (Pares *et al.*, 1996). Il pourrait être impliqué dans la reconnaissance des dipeptides D-alanyl-D-alanine utilisés dans la construction des couches de peptidoglycane de la paroi cellulaire (Yeats *et al.*, 2002). Ces motifs sont aussi retrouvés dans le domaine extracellulaire de PrkC chez *B. subtilis* (Madec *et al.*, 2002) et dans celui de PknB chez *M. tuberculosis* (Young *et al.*, 2003), suggérant un rôle de ces kinases dans la surveillance de l'état du peptidoglycane.

Chez *M. xanthus*, PktB8 associée à PktA5, protéine-kinase dont le domaine C-terminal est inconnu, joue un rôle dans le développement de la sporulation en interagissant avec EspA et EspB, respectivement une histidine kinase senseur et un transporteur de peptide putatif (Stein *et al.*, 2006).

D'autres domaines couplés au domaine kinase des Ser/Thr kinases de type Hanks chez les bactéries ont été identifiés (Krupa & Srinivasan, 2005). Récemment, différentes Ser/Thr protéine-kinases ont été impliquées dans un certain nombre de processus cellulaires, comme dans la différenciation morphologique et le métabolisme secondaire (Matsumoto *et al.*, 1994 ; Umeyama *et al.*, 2002 ; Nariya & Inouye, 2005a), la réponse au stress oxydant (Neu *et al.*, 2002), la biosynthèse des purines chez *Streptococcus agalactiae* (Rajagopal *et al.*, 2005), la biosynthèse des acides mycoliques chez *M. tuberculosis* (Molle *et al.*, 2006a), la consommation du glycogène et le

métabolisme du glucose chez *Myxococcus xanthus* (Nariya & Inouye, 2002, 2003, 2005b), la croissance cellulaire et la formation des septa de division (Treuner-Lange *et al.*, 2001 ; Deol *et al.*, 2005 ; Jin & Pancholi, 2006 ; Fernandez *et al.*, 2006 ; Thakur & Chakraborti, 2006), et la virulence chez *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis* et *M. tuberculosis* (Cozzone, 2005 ; Krupa & Srinivasan, 2005). On peut également noter que, chez les cyanobactéries, les Ser/Thr protéine-kinases sont aussi impliquées dans la motilité cellulaire, la réponse au stress, le contrôle du métabolisme du carbone, la différenciation cellulaire, le métabolisme de l'azote et le rythme circadien (Zhang *et al.*, 2005).

En ce qui concerne les Tyr protéine-kinases de type Hanks, seulement 2 exemples ont été décrits chez les bactéries. Il s'agit de WaaP de *Pseudomonas aeruginosa* qui possède, en plus de son activité protéine-kinase, une activité kinase sur les sucres qui est essentielle pour la biosynthèse des lipopolysaccharides (Zhao & Lam, 2002), et de MasK, une Tyr protéine-kinase essentielle de *M. xanthus*, qui interagit avec MglA, une GTPase essentielle pour le contrôle de la motilité et du développement (Thomasson *et al.*, 2002).

1.1.2.2 Les autres Ser/Thr/Tyr protéine-kinases

Les Ser/Thr kinases

D'autres Ser/Thr protéine-kinases ne présentant aucune homologie avec les protéine-kinases de type Hanks ont été détectées chez les procaryotes. Par exemple, chez *Bacillus subtilis*, une famille de Ser/Thr protéine-kinases, dite à motif histidine-kinase, a été identifiée. Elle est composée des protéines SpoIIAB, RsbW et RsbT. SpoIIAB est impliquée dans la régulation de la sporulation (Schmidt *et al.*, 1990), RsbW et RsbT sont, quant à elles, impliquées dans la réponse au stress (Boylan *et al.*, 1992 ; Wise & Price, 1995). Les membres de cette famille possèdent des motifs conservés, retrouvés chez les His protéine-kinases des systèmes à deux composants (cf. chapitre 1.1.1) (Kang *et al.*, 1998). Les Ser protéine-kinases possèdent les motifs N, G1 et G2 sur les cinq motifs (H, N, G1, F et G2) conservés chez les histidine-kinases (Figure n°4). Leur mécanisme catalytique précis n'a pas encore été identifié, mais il semble différent de celui des His protéine-kinases des systèmes à deux composants.

Un autre membre de cette famille a été détecté chez *Synechocystis*, Slr1861, qui phosphoryle *in vitro* la protéine Slr1856 sur le résidu Ser54. La protéine codée par le gène adjacent à Slr1861, Slr1860 ou également désignée IcfG, qui participe à la régulation du carbone inorganique et au métabolisme du glucose, est capable de déphosphoryler la protéine Slr1856. IcfG est homologue

aux protéine-phosphatases SpoIIE, RsbU et RsbX de *B. subtilis*, qui agissent en couple avec les Ser protéine-kinases à motif histidine kinase SpoIIAB, RsbW et RSbT, respectivement (Shi *et al.*, 1999).

Les enzymes bifonctionnelles

Deux enzymes comportant une activité sérine protéine-kinase couplée à une activité phosphatase ou phosphorylase ont été caractérisées. La première est l'isocitrate déshydrogénase kinase/phosphatase (AceK) qui catalyse les réactions de phosphorylation et de déphosphorylation de l'isocitrate déshydrogénase (LaPorte & Chung, 1985). Chez *E. coli*, la phosphorylation et déphosphorylation sur le résidu Ser113 du site actif de l'isocitrate déshydrogénase (IDH) régule le flux métabolique entre le cycle de Krebs et le shunt du glyoxylate (Cozzone, 1998b). Cette enzyme ne montre aucune similarité avec les protéine-kinases connues, mais possède un site de liaison à l'ATP. Une mutation de la Lys336 dans ce motif réduit les deux activités, kinase et phosphatase (Miller *et al.*, 1996). Cette dernière est absolument dépendante de la présence d'ATP et d'ADP. De plus, AceK possède une activité ATPase. Des études cinétiques et de mutagenèse aléatoire ont montré que ces trois mécanismes sont catalysés au niveau d'un site actif unique. La réaction phosphatase résulte d'un retour en arrière de l'activité kinase étroitement couplée à une hydrolyse d'ATP (LaPorte, 1993 ; Miller *et al.*, 2000). Ces activités sont inhibées par la fixation de NADPH et de l'isocitrate sur l'IDH. Certains résultats ont permis de supposer que les deux enzymes, AceK et IDH, forment un complexe dans lequel IDH doit avoir une conformation ouverte, permettant l'accès de la sérine au site actif.

La seconde enzyme bifonctionnelle est l'HPr kinase/phosphorylase (HPrK/P) qui régule le rapport HPr/P-Ser-HPr. Chez *B. subtilis*, la répression catabolique est gouvernée par le niveau de phosphorylation du résidu Ser46 de HPr, protéine du système de phosphotransfert phosphoenopyruvate carbohydrate (PTS), et de son homologue Crh (45% d'identité). Les phosphorylations de HPr et Crh sont catalysées par l'HPrK/P. La phosphorylation de HPr sur le résidu Ser46 inhibe celle de l'His15 et joue un rôle régulateur important dans la répression catabolique (Deutscher *et al.*, 1997). La phosphorylation ATP-dépendante de HPr a d'abord été mise en évidence chez *Staphylococcus pyogenes* (Deutscher & Saier, 1983). L'enzyme a ensuite été purifiée chez *Streptococcus faecalis* (Deutscher *et al.*, 1985), puis décrite et purifiée chez d'autres firmicutes, comme *Lactobacillus casei*, *Lactococcus lactis* ou *B. subtilis* (Boel *et al.*, 2003). Les structures d'HPrK/P de *Staphylococcus xylosus*, *Mycoplasma pneumoniae* et *L. casei* (forme tronquée) ont été résolues et montrent que l'enzyme forme un hexamère formé de deux couches de trimères (Fieulaine *et al.*, 2001 ; Marquez *et al.*, 2002 ; Allen *et al.*, 2003). Le site actif de l'HPrK/P se trouve dans la partie C-terminale qui est orientée vers le centre de l'hexamère fonctionnel. Quant

à la partie N-terminale, elle est orientée vers l'extérieur de l'hexamère et sa présence n'est pas requise pour l'activité catalytique (Fieulaine *et al.*, 2001). Le motif Walker A (GxxxxGK(T/S)), site de fixation de l'ATP, participe à la catalyse de la réaction kinase ATP-dépendante, stimulée par la présence de fructose-1,6-bisphosphate. La réaction inverse, liée à l'activité phosphorylase, dépend aussi du motif Walker A. Le Pi se fixe au site Walker A en partie grâce à un contact entre la Lys157 du Walker A, mais il est également maintenu par les Arg202 (du motif conservé ExRGxG) et Arg267 (du motif P(V/I)xxGR(N/K)) au moyen d'une molécule d'eau. Le Pi ainsi stabilisé au site Walker A provoque une attaque nucléophile du groupement phosphate de P-SerHPr (Fieulaine *et al.*, 2002 ; Mijakovic *et al.*, 2002 ; Marquez *et al.*, 2002). Du pyrophosphate est alors formé ; ce dernier est hydrolysé par la pyrophosphatase codée par le gène *yvoE*, en opéron avec *hprK*. L'HPrK/P est la première enzyme connue qui catalyse la réaction de déphosphorylation d'une phosphoprotéine via un mécanisme de phosphorylation. Cette réaction est réversible et, à concentration élevée de pyrophosphate, HPrK/P peut utiliser ce dernier pour phosphoryler HPr.

Les Tyr protéine-kinases

La première caractérisation génétique et biochimique d'une Tyr protéine-kinase bactérienne, Ptk chez *Acetobacter Johnsonii*, date de 1997 (Grangeasse *et al.*, 1997). Ptk est localisée dans la membrane cytoplasmique de cet organisme. En présence d'ATP, elle est capable de s'autophosphoryler *in vitro* sur plusieurs résidus de tyrosine (Duclos *et al.*, 1996 ; Grangeasse *et al.*, 1997). Depuis, d'autres Tyr protéine-kinases homologues à Ptk ont été caractérisées chez les actinobactéries, firmicutes, et proteobactéries (Ilan *et al.*, 1999 ; Vincent *et al.*, 2000). L'analyse des séquences de ces Tyr protéine-kinases n'a pas montré d'homologie claire avec les protéine-kinases de type Hanks, suggérant que Ptk et ses homologues procaryotes utilisent un mécanisme différent et encore inconnu (Ilan *et al.*, 1999). Cependant, elles sont bien conservées chez les procaryotes et ont été regroupées dans une nouvelle famille, les BY-kinases (Bacterial Tyrosine Kinases) (Grangeasse *et al.*, 2007).

Structuralement, elles sont composées d'un domaine transmembranaire et d'un domaine catalytique intracellulaire (Doublet *et al.*, 2002 ; Morona *et al.*, 2000 ; Mijakovic *et al.*, 2003). La mise en place de cette organisation diffère entre les protéobactéries/actinobactéries, d'une part, et les firmicutes de l'autre (Soulat *et al.*, 2006). Chez les protéobactéries/actinobactéries, ces deux domaines sont liés et forment un seul polypeptide. Chez les firmicutes, ces deux domaines communiquent grâce aux interactions spécifiques de leurs hélices (Figure n°5). Plusieurs études ont montré l'influence du domaine transmembranaire sur l'activité kinase chez les firmicutes (Morona *et al.*, 2000 ; Bender *et al.*, 2003 ; Mijakovic *et al.*, 2003 ; Soulat *et al.*, 2006), suggérant un rôle de domaine senseur, bien qu'aucun signal ne soit actuellement connu. Cependant, à part quelques

exceptions, il n'y pas d'influence du domaine transmembranaire sur le domaine catalytique chez les protéobactéries (Wugeditsch *et al.*, 2001 ; Grangeasse *et al.*, 2002). Le site actif est défini par les motifs classiques Walker A et B et un motif Walker A' additionnel (Figure n°5). Le motif Walker A' n'est pas communément trouvé chez les protéines se fixant aux nucléotides, mais il apparaît chez certaines kinases phosphorylant des substrats de faible poids moléculaire, comme le shikimate ou le gluconate, ainsi que chez certaines GTPases de la classe des SMIBI (after signal recognition particule, MinD, and BioD) (Leipe *et al.*, 2003). Pour Ptk et Wzc, une BY protéine-kinase identifiée chez *E. coli* (Vincent *et al.*, 1999), le motif Walker A s'est révélé essentiel pour l'autophosphorylation de ces protéines sur des résidus Tyr, suggérant un nouveau mécanisme de phosphorylation. L'autophosphorylation s'effectue au niveau d'un cluster de tyrosines C-terminal (Figure n°5). La position et le nombre de tyrosines ne sont pas conservés et cette région est entourée de glycines, permettant une bonne exposition des chaînes latérales des tyrosines. Six sites de phosphorylation de Wzc d'*E. coli* K12 ont été identifiés (par prédiction puis mutagénèse dirigée), dont cinq sont localisés dans la série de tyrosines à l'extrémité C-terminale et le sixième en amont de ce groupe de tyrosines, situé près du motif Walker A'. Il a été montré que l'autophosphorylation de ce résidu, conservé exclusivement chez les protéobactéries, provoque une stimulation de l'activité kinase de Wzc, qui peut ensuite phosphoryler les cinq autres tyrosines par un processus intermoléculaire (Grangeasse *et al.*, 2002). Il semble que ce soit le niveau de phosphorylation globale de la région qui importe pour le rôle biologique des BY protéine-kinases (Paiment *et al.*, 2002), modulant probablement la force des interactions avec d'autres protéines (Collins *et al.*, 2006).

Au niveau fonctionnel, les Tyr protéine-kinases sont impliquées dans divers processus cellulaires. La phosphorylation au niveau des résidus tyrosyles chez les bactéries n'est pas restreinte exclusivement aux BY-kinases ou aux protéine-kinases de type eucaryote. La protéine DivL de *Caulobacter crescentus*, qui appartient à un système classique à deux composants (cf chap 1.1.1), est autophosphorylée de manière inattendue sur une tyrosine (Wu *et al.*, 1999). Cette tyrosine phosphorylée est localisée à la place de l'histidine qui est habituellement autophosphorylée. Un autre exemple est une protéine de la famille des guanidino-phosphotransférases, la protéine McsB de *Bacillus subtilis*. Elle possède un domaine guanidino-phosphotransférase comme site actif. McsB est une autokinase qui exige l'interaction avec McsA pour sa pleine activité et ressemble sur ce point aux BY-kinases des firmicutes (Kirstein *et al.*, 2005).

Très récemment, une tyrosine protéine-kinase a été identifiée chez *M. tuberculosis*. Alors qu'aucune activité tyrosine kinase n'avait encore clairement été mise en évidence chez cet organisme, Av-Gay et ses collaborateurs ont décrit une nouvelle protéine kinase, nommée PtkA,

capable de phosphoryler la protéine PtpA sur des résidus tyrosyles. De manière surprenante, PtkA ne présente aucun motif conservé homologue aux autres tyrosine kinases déjà étudiées (Bach *et al.*, 2009).

1.2 *MYCOBACTERIUM TUBERCULOSIS*

A l'échelle mondiale, les maladies infectieuses restent les premières causes de mortalité et de morbidité, devant les maladies cardio-vasculaires et les cancers (www.who.int).

Parmi ces maladies infectieuses, la tuberculose, dont l'agent étiologique est *Mycobacterium tuberculosis*, est une maladie pulmonaire qui demeure en expansion constante. La mise en place de nouvelles stratégies de prévention et de lutte contre les mycobactérioses est un enjeu mondial majeur.

Cent vingt-cinq ans après la découverte du bacille de Koch, la tuberculose demeure une maladie importante au niveau mondial puisqu'un tiers de la population mondiale est infectée par le bacille sous forme latente (Check, 2007). En 1993, l'Organisation Mondiale de la Santé (OMS) a déclaré l'état d'urgence contre la tuberculose. L'OMS estime que 1,7 million de personnes sont mortes de la tuberculose et 9 millions de nouveaux cas ont été déclarés en 2004. Les populations les plus affectées sont celles des pays en voie de développement. En France, le nombre de cas de tuberculose déclarés était de 5 374 soit 8,9 cas pour 100 000 habitants, en 2004.

Les efforts conjoints des campagnes d'information, de vaccination et de dépistage ont réussi à faire reculer la maladie pendant toute la durée du XX^{ème} siècle. En revanche, de nos jours cette maladie est considérée comme ré-émergente, infectant 8 à 9 millions de personnes et tuant près de trois millions de personnes par an sur la planète (Hernandez *et al.*, 2008).

La ré-émergence de cette infection s'explique par l'augmentation de la pauvreté et de la précarité de par le monde (Frieden *et al.*, 2003). Maladie pouvant être opportuniste, la réapparition de la tuberculose est historiquement et géographiquement concomitante avec les épidémies de SIDA (Dye, 2006). En effet, différents facteurs peuvent favoriser le risque de contracter ou de développer la tuberculose. Parmi ces facteurs, la co-infection avec le virus de l'immunodéficience humaine (VIH) forme une association dévastatrice. D'après l'OMS, la tuberculose est responsable d'environ 13% des décès des personnes atteintes du syndrome d'immunodéficience acquise (SIDA) dans le monde.

D'autres facteurs tendent à amplifier ce phénomène, comme le suivi et la durée des traitements (6 mois) et l'apparition de souches multi-résistantes au traitement (MDR/XDR) (Hernandez *et al.*, 2008).

1.2.1. Dépistage, traitement et prévention de la tuberculose

1.2.1.1 Dépistage

La tuberculose présente des signes cliniques non spécifiques comme la toux persistante, des douleurs dans la poitrine, de la fièvre, un état d'affaiblissement, un amaigrissement et des sueurs nocturnes. Différents moyens de dépistage clinique et bactériologique de la maladie sont disponibles:

- un examen microscopique, réalisé à partir d'expectorations dans le cas de tuberculose pulmonaire, pour identifier le bacille tuberculeux. Cette méthode, qui date de 120 ans, est peu sensible.
- des colorations du bacille tuberculeux particulières à partir d'expectorations de patients, comme la coloration de Ziehl-Neelsen qui utilise les propriétés acido-alcooloresistantes de la bactérie (Shoub, 1923). Ce moyen de dépistage, qui date de plus de cent ans, présente l'inconvénient de diagnostiquer de nombreux faux positifs.
- l'utilisation de rayons X, mais ils s'avèrent non adaptés dans le cas de la tuberculose extra-pulmonaire.

Ces moyens de diagnostic ne sont utilisables que dans les cas de tuberculose active, ce qui représente seulement 10% des personnes infectées par le bacille de Koch. Dans les 90% de cas restants, qui présentent une tuberculose en phase latente, seul le test à la tuberculine est possible. La tuberculine est une fraction protéique de *M. tuberculosis* qui, injectée en sous-cutané, provoque un oedème au point d'injection dans le cas d'un test positif, du fait de l'accumulation de lymphocytes mémoires sensibilisés lors de la primo-infection. Ce test présente cependant des limites puisque la vaccination par le vaccin du BCG (Bacille Calmette et Guérin) peut conduire à une réaction du même type.

1.2.1.2 Le traitement antibiotique

Les premiers agents antituberculeux ont été découverts dans les années 1950-1960, l'isoniazide et la pyrazinamide en 1952, l'éthambutol en 1961 et la rifampicine en 1966.

Les monothérapies ont rapidement montré leurs limites avec l'apparition de souches résistantes. En 2006, les équipes de Médecins sans Frontières (MSF) ont soigné 20 000 malades de

la tuberculose dans plus de 40 pays. Parmi eux, plusieurs souffraient de formes multirésistantes (MDR-TB) et ultrarésistantes (XDR-TB) (www.msf.dr). La tuberculose multirésistante est due à une infection par des bacilles résistants aux antibiotiques dits de première classe comme la rifampicine et l'isoniazide qui sont les deux antibiotiques majeurs dans le traitement contre la tuberculose. La tuberculose ultrarésistante est également une forme de tuberculose résistante au traitement par les antibiotiques de première classe et les antibiotiques dits de seconde classe appartenant à la famille des fluoroquinolones et à la famille des aminoglycosides (comme la kanamycine et l'amikacine). Entre 2000 et 2004, 20% des souches responsables de la tuberculose dans le monde étaient résistantes aux antibiotiques de première classe et un dixième d'entre elles étaient ultrarésistantes (www.msf.fr).

Actuellement, le traitement classique de la tuberculose consiste en une thérapie de quatre antibiotiques pendant deux mois (isoniazide, rifampicine, pyrazinamide et éthambutol) suivie d'une bithérapie pendant les quatre mois suivants (isoniazide et rifampicine).

➤ *L'isoniazide* est un dérivé de l'acide isonicotinique. La cible majeure de ce composé est le système de synthèse des acides gras appelé Fatty Acid Synthase II (FAS II). Ce système intervient dans la synthèse des acides mycoliques. Cet antituberculeux est le composé le plus prescrit. Cependant, il existe des souches de mycobactéries résistantes à l'isoniazide (Somoskovi *et al.*, 2001).

➤ *La rifampicine* est un composé semi-synthétique issu de la rifamycine B. Ce médicament inhibe spécifiquement l'ARN polymérase ADN-dépendante, enzyme responsable de la transcription bactérienne (Somoskovi *et al.*, 2001). Cet antibiotique ne présente aucune action sur les ARN polymérases humaines. La prescription de cet antituberculeux a largement contribué à la diminution des traitements à six mois. Comme pour l'isoniazide, il existe des souches résistantes à cet antibiotique (Somoskovi *et al.*, 2001).

➤ *Le pyrazinamide* est un analogue du nicotinamide. Cet antituberculeux a un pouvoir bactéricide uniquement en milieu acide. La cible de ce composé est le système de synthèse des acides gras de type I (FAS I), impliqué dans la synthèse des acides gras du bacille (Zimhony *et al.*, 2000). Il existe également des souches mycobactériennes résistantes à cet antibiotique (Lemaitre *et al.*, 1999 ; Louw *et al.*, 2006).

➤ *L'éthambutol* est un inhibiteur spécifique de la synthèse de l'arabinogalactane qui entre dans la composition de l'enveloppe de la bactérie. Cet antibiotique cible plus précisément les arabinosyltransférases impliquées dans la formation de l'arabinogalactane (Takayama & Kilburn, 1989). Des souches résistantes à cet antibiotique présentent des mutations au niveau des arabinosyltransférases (Ramaswamy *et al.*, 2000).

1.2.1.3 La prévention

Le seul vaccin disponible à ce jour est le BCG. Cette souche vaccinale a été obtenue à partir d'une souche virulente de *M. bovis* qui, après 230 passages sur des extraits de pomme de terre glycélinés contenant de la bile de bœuf, ne provoquait plus la tuberculose chez les bovins, même à forte dose, et protégeait les animaux soumis à une présentation de bacilles virulents. En 1921, le premier essai de vaccination d'un jeune enfant a eu lieu avec succès. A partir de 1924, la vaccination systématique par le BCG a été réalisée en Europe et dans le monde.

L'efficacité du vaccin BCG est comprise entre 75 et 85% contre les formes pulmonaires du nourrisson et du jeune enfant. Chez l'adulte, l'efficacité varie entre 30 et 50%. Ce vaccin ne permet donc pas d'empêcher la transmission de la maladie, ni d'enrayer l'épidémie mondiale.

En 1949, la France a adopté le projet de loi sur l'obligation de la vaccination par le BCG. En juin 2004, seule la primo-vaccination demeurait obligatoire avant l'entrée en collectivité de l'enfant ou dans le cadre d'une profession comportant un risque pour l'adulte. Compte tenu de l'efficacité relative de ce vaccin, de ses effets indésirables nombreux et importants (dermatoses, bécégites qui peuvent s'avérer mortelles) ainsi que le faible taux d'incidence de la tuberculose en France, la vaccination généralisée chez l'enfant a été suspendue en juillet 2007.

Depuis l'apparition des premiers traitements dans les années 1950-1960, aucun nouvel agent antituberculeux n'a été découvert (www.msf.fr). L'absence de développement de nouveaux antituberculeux, associée au problème de multirésistance des souches, relance la nécessité de développer de nouvelles molécules antimycobactériennes. De plus, l'efficacité relative de la protection par le BCG, le seul vaccin disponible actuellement, nécessite le développement de nouvelles stratégies vaccinales.

L'atteinte de ces objectifs passe par une meilleure compréhension de la physiopathologie des infections mycobactériennes.

1.2.2. *M. tuberculosis*, l'agent étiologique de la tuberculose humaine

1.2.2.1 Le genre *Mycobacterium*

Le genre *Mycobacterium* appartient à la famille des *Mycobacteriaceae* qui fait partie du sous-ordre des *Corynebacterineae* dans l'ordre des actinomycètes. Les bactéries du genre *Mycobacterium* sont aérobies, asporulées, immobiles, à Gram-positif, et ont la particularité d'être riches en lipides (30 à 60 % de la masse sèche de leur enveloppe). Une des propriétés des

Corynebacterineae est de produire des acides gras particuliers: les acides mycoliques. Ces acides gras alpha-ramifiés et bêta-hydroxylés peuvent être de longueur variable selon le genre et l'espèce (Shinnick & Good, 1994).

Le genre *Mycobacterium* regroupe une soixantaine d'espèces dont la plupart sont des organismes saprophytes nichant dans le sol. Ce genre se distingue aussi par sa propriété d'acido-alcool résistance. Les mycobactéries colorées à la fuschine phéniquée retiennent le colorant après traitement à l'alcool tandis que les autres bactéries sont rapidement décolorées. Cette propriété est à la base de la coloration des mycobactéries par la technique de Ziehl-Neelsen (Shoub, 1923). Le génome des mycobactéries présente un fort taux de guanines et cytosines (environ 60 à 70%) (Brennan & Nikaido, 1995).

Les mycobactéries peuvent être classées selon leur vitesse de croissance. Les bacilles à croissance rapide, comme *M. smegmatis*, ont un temps de génération de 2 à 5h alors que les bacilles à croissance lente, comme *M. tuberculosis*, ont un temps de génération de 24h environ.

Plusieurs mycobactéries sont pathogènes. Les mycobactéries appartenant au complexe *tuberculosis* causent la tuberculose : *M. tuberculosis*, *M. africanum*, *M. canetti*, *M. bovis* et *M. microti*. La tuberculose chez l'Homme est provoquée par *M. tuberculosis* et *M. africanum*. Quelques cas de tuberculose par infection avec *M. canetti* ont été décrits (van Soolingen *et al.*, 1997). *M. microti* est pathogène chez les rongeurs et *M. bovis* est responsable de la tuberculose des bovidés. Deux autres mycobactéries sont responsables de maladies tristement célèbres : *M. ulcerans* cause les ulcères de Buruli, et *M. leprae* est l'agent étiologique de la lèpre.

1.2.2.2 Le cycle infectieux

La transmission de la tuberculose se fait par voie aérienne. Quand une personne malade tousse, elle expulse des milliers de minuscules gouttelettes qui peuvent contenir le bacille tuberculeux. Une fois inhalés, les germes se propagent dans le système respiratoire et pénètrent dans les poumons au niveau des alvéoles pulmonaires où ils rencontrent les macrophages alvéolaires qui les phagocytent. *M. tuberculosis* a la capacité de survivre et de se multiplier au sein de ces macrophages. L'infection des macrophages engendre une réponse pro-inflammatoire locale, induisant en particulier la sécrétion de chimiokines et de cytokines qui vont permettre le recrutement d'autres cellules de la réponse immunitaire pour aboutir à la formation d'une structure multicellulaire, le granulome, également appelé tubercule ou tuberculoma (terme principalement utilisé par les cliniciens) caractéristique de la tuberculose (Russell, 2007). Le granulome est constitué de macrophages infectés, entourés de phagocytes mononucléés, puis d'un manteau de lymphocytes associés à une gangue fibreuse contenant du collagène et d'autres composés matriciels

qui délimitent la périphérie de cette structure (Figure n°6). Le granulome ainsi formé est capable de contenir l'infection durant des années (Ulrichs & Kaufmann, 2006). A des stades tardifs, la taille de la gangue fibreuse est importante et le nombre de vaisseaux sanguins pénétrant la structure diminue ; le centre du granulome est alors en hypoxie (Tsai *et al.*, 2006). Suite à un changement de l'état du système immunitaire de l'hôte, lié par exemple à l'âge, la malnutrition ou encore une co-infection avec le VIH, les macrophages infectés par les mycobactéries se lysent. Les bacilles se propagent et se disséminent à travers l'organisme et l'hôte développe alors une tuberculose active (Figure n°6). Seules 10% des personnes infectées par le bacille tuberculeux développent la tuberculose et cette maladie est létale dans 50% des cas (Kaufmann, 2001).

1.3 LES PROTÉINE-KINASES DE *MYCOBACTERIUM TUBERCULOSIS*

Chez les mycobactéries, la transduction du signal s'opère à la fois grâce à la présence de systèmes à deux composants et à l'existence de Ser/Thr protéine-kinases (STPKs).

Le séquençage de l'intégralité du génome de *Mycobacterium tuberculosis* a mis en évidence 11 systèmes à deux composants ainsi que 11 STPKs (Cole *et al.*, 1998 ; Ponting *et al.*, 1999 ; Av-Gay & Everett, 2000). Il est intéressant de noter que le nombre de STPKs varie parmi les mycobactéries. Ainsi *M. leprae* n'en compte que quatre dans son génome alors que *M. marinum* en compte 24 (Wehenkel *et al.*, 2008).

Toutes les STPKs de *M. tuberculosis* présentent les onze sous-domaines de Hanks caractéristiques des « eukaryotic-like » STPKs (Wehenkel *et al.*, 2008). Parmi les onze STPKs identifiées chez *M. tuberculosis*, neuf présentent un domaine transmembranaire et deux semblent être cytoplasmiques (PknG et PknK) (Cole *et al.*, 1998) (Figure n°7). Les neuf STPKs membranaires sont subdivisées en trois groupes basés sur des similarités de séquence peptidique: PknA/PknB/PknL, PknD/PknE/PknH, et PknF/PknI/PknJ (Av-Gay & Everett, 2000 ; Narayan *et al.*, 2007).

1.3.1 Groupe 1: PknA/B/L

1.3.1.1 PknA

Chez *M. tuberculosis*, les gènes *pknA* et *pknB* codant les kinases PknA et PknB sont situés dans un cluster de gènes contenant également les gènes *pbpA* et *rodA* impliqués dans la biosynthèse des composants de la paroi cellulaire, ainsi que *mstP*, gène codant la sérine/thréonine phosphatase de *M. tuberculosis* (Cole *et al.*, 1998). Le fait que les différents gènes faisant partie de cet opéron

soient conservés chez les espèces mycobactériennes pathogènes et les non-pathogènes et, d'autre part, que les gènes *pknA* et *pknB* soient essentiels (Kang *et al.*, 2005) semble montrer que leurs fonctions sont indispensables pour la survie de la bactérie (Sasseti *et al.*, 2003 ; Narayan *et al.*, 2007).

Les travaux menés par l'équipe de Chakraborti ont permis la caractérisation biochimique de PknA et la mise en évidence de son activité d'autophosphorylation aux dépens de l'ATP. PknA est une protéine de 431 acides aminés dont le domaine catalytique se situe dans la portion intracellulaire. Ce domaine kinase est lié au domaine extracellulaire par une hélice transmembranaire. Bien que la structure de PknA ne soit pas encore résolue, l'étude menée par Thakur *et al.* en 2008 montre que, contrairement à PknB et PknF, le domaine kinase actif de cette protéine est composé non seulement du domaine catalytique classique, mais également de la région juxtamembranaire reliant le domaine catalytique à l'hélice transmembranaire. Les auteurs démontrent l'importance, dans le mécanisme d'autophosphorylation de PknA, de la phosphorylation des résidus Thr172 et Thr174 situés dans la boucle d'activation. De plus, l'autoactivation de cette kinase pourrait se faire par le biais d'un mécanisme d'interphosphorylation bimoléculaire (Thakur *et al.*, 2008).

Les premières expériences de surexpression de PknA ont mis en évidence, à la fois chez *E. coli* et chez *Mycobacterium smegmatis*, un allongement des cellules surproductrices, pointant un rôle possible de la kinase dans les mécanismes de régulation de la forme de la cellule, ou encore dans la formation du septum lors de la division cellulaire (Chaba *et al.*, 2002 ; Kang *et al.*, 2005).

L'analyse par électrophorèse bi-dimensionnelle couplée au Western-blotting d'un lysat cellulaire a permis d'identifier Wag31 comme étant phosphorylée chez *M. tuberculosis*. Aussi connu sous le nom de Ag84 ou DivIVA, Wag31 est une protéine essentielle de *M. tuberculosis* impliquée dans la régulation de la forme cellulaire et la formation du septum (Kang *et al.*, 2005). La surexpression de Wag31 entraîne un phénotype singulier incluant une forme cellulaire aberrante accompagnée d'une dérégulation de la formation du septum de division (Nguyen *et al.*, 2007). La phosphorylation *in vivo* de Wag31 a été confirmée par l'analyse en Western blotting de souches surexprimant Wag31. De plus, des tests de phosphorylation *in vitro* ont révélé que PknA phosphorylait Wag31 sur le résidu Thr73. Les différences phénotypiques observées lors de l'expression *in vivo* de Wag31 ainsi que des mutants T73A (non phosphorylables), et T73E (mimant l'état phosphorylé), semblent indiquer que l'état de phosphorylation de Wag31 est déterminant pour le contrôle de la forme cellulaire (Kang *et al.*, 2005).

La protéine FtsZ a également été identifiée comme un substrat de PknA. Cette GTPase correspond à l'homologue bactérien de la tubuline eucaryote et contribue à la formation du septum

lors de la division bactérienne. De manière intéressante, la phosphorylation de FtsZ par PknA abolit son activité GTPase et empêche ainsi sa polymérisation (Thakur & Chakraborti, 2006).

Récemment, il a été montré que PknA phosphorylait MurD aussi bien *in vitro* qu'*in vivo* (Thakur & Chakraborti, 2008). Cette D-glutamate ligase catalyse la réaction d'addition du D-glutamate sur l'UDP-N-acétylmuramoyl-L-alanine au sein de la voie de biosynthèse du peptidoglycane. La division cellulaire et la synthèse du peptidoglycane sont deux mécanismes différents mais corrélés, et il semblerait donc que PknA ait un rôle clé dans ces voies métaboliques.

Il a aussi été montré que PknA était capable de phosphoryler des enzymes impliquées dans les voies de biosynthèse des acides mycoliques, composants de la paroi cellulaire (Molle *et al.*, 2006a). Ces substrats, KasA et KasB, interviennent au niveau des étapes de condensation lors de la synthèse de β -hydroxy-acide gras au sein du système FAS-II (voie d'élongation des acides gras). La protéine *mtFabD*, un autre composant du système Fas-II dont le gène correspondant se situe dans le même opéron, est également phosphorylée par PknA. Il faut par ailleurs mentionner que ces différents substrats peuvent être phosphorylés par PknB, PknE, PknF et PknH (Molle *et al.*, 2006b), mettant ainsi à jour la complexité des voies de signalisation régies par les STPKs.

Il est intéressant de rappeler ici qu'il existe chez *M. tuberculosis* une unique Ser/Thr phosphatase, PstP, dont le gène est situé sur le même opéron que PknA. D'une manière générale, les Ser/Thr phosphatases déphosphorylent spécifiquement les résidus P-séryles et P-thréonyles. Ainsi, il a été montré que PstP déphosphorylait efficacement PknA, suggérant un rôle régulateur de cette Ser/Thr phosphatase dans l'activité de la kinase (Chopra *et al.*, 2003). Le fait que les gènes codant la kinase et la phosphatase soient sur le même opéron laisse à imaginer une régulation potentielle entre les deux protéines (Boitel *et al.*, 2003 ; Chopra *et al.*, 2003).

L'ensemble de ces données tend à démontrer le rôle de PknA dans la régulation de la division cellulaire et dans le contrôle de la forme de la bactérie.

1.3.1.2 PknB

Le gène *pknB* est conservé chez toutes les espèces de *Mycobacterium* et correspond à un gène essentiel chez *M. tuberculosis* et *M. smegmatis*. L'expression de *pknB* augmente de près de dix fois lorsque la bactérie entre en phase stationnaire. De plus, la surexpression ou la délétion partielle de ce gène entraîne de sévères changements morphologiques ainsi qu'une viabilité réduite des bacilles, suggérant un rôle possible de PknB dans le contrôle de la forme cellulaire (Kang *et al.*, 2005).

D'un point de vue structural, PknB présente l'organisation caractéristique des kinases dites de Hanks. Le domaine extracellulaire comporte 4 séquences répétées de 66 à 68 aminoacides

nommées « penicillin A binding and Ser/Thr kinase attached » (domaine PASTA) (Yeats *et al.*, 2002). Ces domaines PASTA sont souvent retrouvés sur les « penicillin binding proteins » procaryotes impliquées dans la synthèse de la paroi bactérienne (Young *et al.*, 2003). D'autre part, la région intracellulaire contient le domaine kinase catalytique. Les deux domaines sont liés entre eux par une hélice transmembranaire et une courte séquence linker de 52 résidus. Le repliement général du domaine kinase se présente sous forme bi-lobée avec un sous-domaine N-terminal composé d'un feuillet β et d'une longue hélice α (hélice C α), et un sous-domaine C-terminal comportant des hélices α (Figure n°8) (Ortiz-Lombardia *et al.*, 2003), avec le nucléotide piégé dans la crevasse créée entre les deux lobes. L'activité d'autophosphorylation de PknB a été mise en évidence aussi bien avec la protéine entière qu'avec uniquement le domaine catalytique (Av-Gay *et al.*, 1999), suggérant que ce domaine suffit à générer une conformation active stable (Boitel *et al.*, 2003). Cinq sites de phosphorylation ont été identifiés dont deux sur les résidus thréonyles T171 et T173 de la boucle d'activation (Boitel *et al.*, 2003 ; Duran *et al.*, 2005). La phosphorylation de cette boucle est primordiale pour l'activation maximale de la kinase ainsi que pour la phosphorylation de ses substrats (Boitel *et al.*, 2003). Contrairement à ce que l'on retrouve chez les kinases eucaryotes, la boucle d'activation de PknB est désordonnée. Néanmoins on retrouve un cluster d'acides aminés chargés positivement, correspondant aux résidus R55, R137 et R161, étant capable de stabiliser la boucle d'activation une fois celle-ci phosphorylée. (Ortiz-Lombardia *et al.*, 2003). De plus, la déphosphorylation de ces deux résidus thréonyles par la PstP entraîne une diminution de l'activité de phosphorylation de PknB (Boitel *et al.*, 2003). L'ensemble de ces observations suggère donc que la phosphorylation de la boucle d'activation est un élément majeur de la régulation de l'activité de la kinase.

Récemment, le domaine catalytique de PknB a été cristallisé en complexe avec de la mitoxantrone, une molécule compétitive de l'ATP utilisée dans le traitement anticancéreux. Ces expériences ont permis de mettre en évidence un phénomène de dimérisation de la kinase. Cette dimérisation s'opère via le lobe N-terminal afin de placer les deux monomères en conformation "dos à dos" (Wehenkel *et al.*, 2006). Il semblerait que la dimérisation soit un mécanisme de régulation de l'activation de la kinase. De plus, la cristallisation du domaine kinase de PknB en présence de mitoxantrone a, en outre, permis de démontrer que l'inhibiteur se place dans une crevasse hydrophobe habituellement occupée par l'ATP sans pour autant en épouser totalement la forme, avec pour conséquence l'inhibition de la kinase et l'arrêt de la croissance des mycobactéries. Cette découverte ouvre ainsi de nouveaux champs d'investigation dans l'identification de nouveaux médicaments anti-tuberculeux.

La recherche de substrats de PknB a permis d'identifier GarA (Rv1827). Au niveau C-terminal, GarA porte un domaine ForkHead-Associated (FHA) (Durocher & Jackson, 2002). Ce

type de domaine forme un « sandwich » de onze feuillets β et est impliqué dans l'interaction des protéines phosphorylées (Durocher & Jackson, 2002). Des tests de phosphorylation associés à la spectrométrie de masse ont permis l'identification d'un site unique de phosphorylation chez GarA : la Thr22. De manière intéressante, un traitement par la PstP ainsi que la mutation des deux sites de phosphorylation situés dans la boucle d'activation de PknB entraînent une diminution de la phosphorylation de GarA, mettant en évidence que le recrutement et la phosphorylation de GarA sont dépendants de l'état d'autophosphorylation et donc de l'activation de PknB (Villarino *et al.*, 2005). Il est cependant important de noter que plusieurs protéines contenant un domaine FHA peuvent être phosphorylées par plusieurs kinases mycobactériennes incluant PknA, PknB, PknD, PknE, PknF, PknG et PknH (Molle *et al.*, 2003b, 2004 ; Grundner *et al.*, 2005 ; Sharma *et al.*, 2006a). Outre GarA, la protéine PBPA (Penicillin Binding Protein A) appartenant au groupe des sérine acyltransférases impliquées dans la synthèse du peptidoglycane (Popham & Young, 2003) a été identifiée comme étant phosphorylée par PknB (Dasgupta *et al.*, 2006). Une mutation du résidu Thr437, site potentiel de phosphorylation de PBPA, entraîne un ralentissement de la croissance ainsi qu'une mauvaise localisation de la protéine normalement retrouvée au niveau du septum, suggérant que la phosphorylation de PBPA par PknB permettrait son adressage au septum (Dasgupta *et al.*, 2006).

Récemment, une autre enzyme impliquée dans la synthèse du peptidoglycane a parallèlement été identifiée comme étant phosphorylée par PknB. En effet, la phosphorylation de la N-acétylglucosamine-1-phosphate uridyltransférase par PknB provoque une diminution de l'activité acétyltransférase de cette enzyme (Parikh *et al.*, 2009).

En conclusion, les différents substrats de PknB identifiés à ce jour tendent à indiquer que cette kinase jouerait un rôle dans la régulation de la synthèse de la paroi bactérienne.

1.3.1.3 PknL

PknL est constituée de 399 acides aminés. Cette kinase diffère des autres STPKs de *M. tuberculosis* du fait que son domaine transmembranaire est placé au niveau de son extrémité C-terminale, et qu'elle ne posséderait pas de domaine extracellulaire. PknL partage avec PknA, B et G le fait d'avoir des homologues chez *M. leprae*, ce qui tend à montrer que cette kinase serait essentielle chez les mycobactéries pathogènes.

De plus, l'analyse du locus génétique de *pknL* dans le génome de *M. tuberculosis* montre que ce gène fait partie d'un cluster de 30 kb portant plusieurs gènes impliqués dans la synthèse de

l'enveloppe bactérienne et la division cellulaire (cluster *dcw*), suggérant ainsi un rôle possible de PknL dans la régulation de ce cluster de gènes (Narayan *et al.*, 2007).

1.3.2 Groupe 2: PknD/E/H

1.3.2.1 PknD

PknD est une protéine transmembranaire de 69 kDa. Chez *M. tuberculosis*, cette kinase est membranaire alors que chez *M. bovis* BCG on observe une forme cytoplasmique de 31 kDa tronquée au niveau de la région transmembranaire ainsi que du domaine régulateur C-terminal (Peirs *et al.*, 2000). La localisation du gène *pknD*, placé entre les gènes *pstS-2* et *pstA-1* codant respectivement une « phosphate-binding protein » et une sous-unité de perméase membranaire à phosphate, pouvait laisser penser que PknD aurait un rôle dans la régulation du transport du phosphate cellulaire. Toutefois, à ce jour, aucune étude n'a pu démontrer cette hypothèse et le mutant PknD montre une activité de récupération du phosphate normale (Peirs *et al.*, 2005).

A l'instar des autres STPKs de *M. tuberculosis*, PknD possède une activité d'autophosphorylation, et les onze sites de phosphorylation identifiés sont localisés à la fois au niveau de la boucle d'activation (Thr169, Thr171 et Thr173) et dans d'autres segments proches du domaine kinase, comme dans le domaine juxtamembranaire (Duran *et al.*, 2005 ; Molle *et al.*, 2006b).

Récemment, MmpL7 correspondant à une protéine de 83 kDa à 12 passages transmembranaires, a été identifiée comme un substrat potentiel de PknD (Perez *et al.*, 2006). Il est à noter qu'il existe 13 gènes codant des protéines de la famille des MmpL chez *M. tuberculosis*, et que plusieurs d'entre eux sont situés à proximité de gènes codant des protéines impliquées dans la synthèse d'acides gras ou de polykétides. En ce qui concerne MmpL7, cette protéine est responsable du transport de phtiocérol dimycocérosérate (PDIM), un important composé de la paroi cellulaire impliqué dans la virulence de *M. tuberculosis* (Perez *et al.*, 2006). Cependant, une interaction directe entre PknD et MmpL7 est peu probable. En effet, les sites phosphoaccepteurs de MmpL7 sont extracellulaires alors que le domaine catalytique de la kinase est cytosolique (Perez *et al.*, 2006).

Dans le but d'élucider le rôle physiologique de PknD, l'expression du gène a été mise sous contrôle d'un promoteur inductible. Ceci a permis de mettre en évidence, dans une souche surexprimant PknD, l'induction significative du gène *rv0516c*, homologue d'un anti-anti facteur sigma. Le site de phosphorylation de Rv0516c par PknD a été identifié comme étant le résidu thréonyle Thr2 (Greenstein *et al.*, 2007).

D'un point de vue structural, peu de choses sont, à ce jour, connues sur les domaines senseurs extracellulaires des STPKs mycobactériennes. Cependant, la prédiction de structure de PknD propose que son domaine C-terminal extracellulaire contienne une hélice β , motif retrouvé chez diverses kinases eucaryotes. Ainsi, la détermination de la structure du domaine senseur de PknD par Good et ses collaborateurs a confirmé que ce domaine forme une hélice β , constituée de six lames arrangées en cercle autour d'un pore central (Figure n°9) (Good *et al.*, 2004). Les lames sont caractérisées par des séquences internes de 40 acides aminés extrêmement conservées par opposition aux boucles, plus variables. Les boucles connectant les lames forment une structure "cup-like" correspondant le plus souvent à une surface fonctionnelle d'interaction.

1.3.2.2 PknE

La caractérisation biochimique de cette kinase a confirmé son autophosphorylation sur de nombreux résidus séryles et thréonyles au sein du domaine catalytique, de la boucle d'activation, ainsi que dans la région juxtamembranaire (Duran *et al.*, 2005; Molle *et al.*, 2003a, 2006b). De manière intéressante, la phosphorylation de sites dans la région juxtamembranaire a également été démontrée chez d'autres kinases mycobactériennes telles que PknB, D et F, indiquant que ces sites semblent être importants pour la stabilité, l'activité ou encore l'interaction avec des partenaires (Duran *et al.*, 2005).

Des études de cristallisation montrent que, comme pour PknB et PknD, le domaine kinase de PknE est présent sous forme d'homodimères placés dos-à-dos. Cependant, alors que le dimère de PknE ressemble globalement au dimère de PknB, certains détails divergent entre les deux structures. D'une part, les acides aminés formant l'interface ne sont pas conservés chez PknE et, d'autre part, la conformation générale de PknE ne ressemble pas à la conformation active canonique des STPKs (Gay *et al.*, 2006).

Le rôle physiologique de PknE reste, à ce jour, à déterminer. Cependant, le promoteur de *pknE* semble répondre au stress nitrique. En effet, en présence de nitroprussiate de sodium (SNP) ou de S-nitroglutathion (GSNO), deux donneurs d'oxyde nitrique, l'activité du promoteur est augmentée. En revanche, l'activité transcriptionnelle se trouve diminuée en réponse au peroxyde d'hydrogène, ce qui tend à montrer que l'expression de *pknE* n'est pas requise lors d'un stress oxydant (Jayakumar *et al.*, 2008). La souche dont le gène *pknE* a été remplacé par une cassette de résistance aux antibiotiques, ne montre pas de différence de croissance, mais une moindre sensibilité au stress nitrique par rapport aux souches sauvages (Jayakumar *et al.*, 2008). Cependant, les souches mutées apparaissent plus sensibles aux agents réducteurs tels que le dithiothréitol (DTT) ou le glutathion réduit, ainsi que vis-à-vis de certains ions métalliques comme le zinc ou le cadmium. La kinase PknE pourrait donc avoir un rôle dans la prise en charge des radicaux libres

d'oxyde nitrique, et pourrait être impliquée dans les mécanismes de résistance lors de l'interaction avec le macrophage. En effet, des tests d'infection du macrophage humain montrent que le taux d'apoptose augmente chez les macrophages infectés par les souches mutantes et cette augmentation est plus marquée si on traite les macrophages avec un donneur d'oxyde nitrique. L'apoptose ayant un effet néfaste sur la survie et la prolifération de *M. tuberculosis* dans l'organisme infecté, il est probable que PknE ait un rôle cytoprotecteur et soit impliquée dans les mécanismes de maintien de la bactérie dans l'hôte après l'infection (Jayakumar *et al.*, 2008).

La sensibilité aux agents réducteurs observée peut s'expliquer par la présence d'un domaine « thioredoxine-like » (motif CXXC) au niveau C-terminal de PknE, ce motif étant impliqué dans des réactions d'oxydo-réduction. On retrouve de tels motifs chez d'autres espèces pathogènes. Par exemple, chez *Neisseria meningitidis*, on retrouve une enzyme disulfure oxydoréductase portant le motif CXXC dans son site actif agissant comme une chaperonne en réduisant ses substrats spécifiques afin de générer un repliement correct et donc leur stabilité (Tinsley *et al.*, 2004). En effet, *M. tuberculosis* est capable de vivre dans des conditions redox variées, passant de conditions aérobies lorsqu'elle n'est pas dans l'hôte à un environnement pauvre en oxygène au sein des granulomes. Ces brusques changements environnementaux requièrent une adaptation rapide et une régulation fine des fonctions protéiques. Par conséquent, la présence de motifs CXXC sur des éléments régulateurs comme les STPKs favoriserait vraisemblablement ces régulations.

Il est, en outre, intéressant de noter que PknE phosphoryle Rv1747, un ABC transporteur qui semble aussi être substrat de PknB, PknD et PknF (Grundner *et al.*, 2005) et semble aussi phosphoryler GarA *in vitro* (Villarino *et al.*, 2005), ceci démontrant, une fois de plus, la complexité des régulations régies par les STPKs mycobactériennes.

1.3.2.3 PknH

De même que les autres STPKs mycobactériennes, PknH possède une activité d'autophosphorylation aux dépens de l'ATP et en présence de cations divalents. L'autophosphorylation de la boucle d'activation et, plus précisément, du résidu Thr170, est nécessaire pour l'activation de la kinase (Molle *et al.*, 2003b). Des analyses de spectrométrie de masse ont permis de mettre en évidence dix sites de phosphorylation correspondant à T36, T41, T48, T170, T174, S219, T222, S291, T307 et T311 (Molle *et al.*, 2006b).

La transcription du gène *pknH* est régulée par le facteur sigma A, principal facteur sigma des mycobactéries. En effet, les conditions d'expression du gène *pknH* montrent que les valeurs basses de pH ainsi que les chocs thermiques (42°C) diminuent l'expression de *pknH*. En revanche, le stress oxydant, l'hypoxie ou la privation de nutriments ne semblent pas affecter la transcription de *pknH*. Par conséquent, le fait que l'expression de *pknH* soit induite sous certaines conditions de stress et,

de plus, que le gène *pknH* soit absent des génomes des espèces non-pathogènes telles que *M. smegmatis*, suggère que PknH pourrait jouer un rôle dans la détection et l'intégration de facteurs de stress en environnement hostile (Sharma *et al.*, 2004).

Le gène *pknH* est situé en aval du gène *embR* codant un activateur transcriptionnel de la famille des SARP (*Streptomyces coelicolor* Antibiotic Regulatory Proteins). EmbR contrôle la transcription de l'opéron *emb*, composé chez *M. tuberculosis* des gènes *embA*, *embB* et *embC* (Sharma *et al.*, 2006b). Les protéines exprimées correspondent aux arabinosyltransférases impliquées dans la synthèse de l'arabinane, précurseur de l'arabinogalactane et du lipoarabinomannane (LAM), composés majeurs de la paroi mycobactérienne et importants facteurs de virulence, notamment pour le LAM. La caractérisation de PknH a montré que cette kinase était capable de phosphoryler EmbR sur plusieurs résidus thréonyles (Molle *et al.*, 2003b).

D'autre part, la structure d'EmbR révèle, au niveau de la région N-terminale, la présence d'un domaine Hélice-Tour-Hélice (HTH) caractéristique des protéines capables de fixer l'ADN. Au niveau de la région C-terminale, on retrouve un domaine d'activation transcriptionnelle, ainsi qu'un domaine de type FHA (Alderwick *et al.*, 2006). Des expériences de délétion et de mutagenèse dirigée ont permis de montrer que la phosphorylation de EmbR par PknH était médiée par le domaine FHA présent sur la protéine EmbR (Molle *et al.*, 2003b). De plus, il a été montré que EmbR était capable de se fixer en amont de l'opéron *embCAB*, ainsi qu'en amont de chaque gène individuellement, et que la phosphorylation d'EmbR avait pour conséquence d'augmenter son affinité pour ces régions d'ADN. Il a été également mis en évidence que EmbR sous sa forme phosphorylée régulait positivement l'expression de l'opéron *emb*.

Comme indiqué précédemment, les produits de l'opéron *embCAB* sont impliqués dans les voies de biosynthèse de composés de la paroi cellulaire. Le rapport LAM/LM est important lors de l'infection des macrophages, car un rapport LAM/LM élevé entraîne une diminution de la réponse immunitaire de l'hôte. La présence de PknH régulant l'expression de l'opéron *embCAB* par le biais de la phosphorylation de EmbR place donc cette kinase au centre d'un mécanisme de virulence lors de l'infection avec l'hôte (Sharma *et al.*, 2006b). Ainsi, afin de caractériser le rôle de PknH, une souche comportant une délétion du gène *pknH* ($\Delta pknH$) a été générée. Cette souche montre une tolérance supérieure aux oxydes nitriques mais une plus grande sensibilité aux peroxydes et aux superoxydes par rapport à la souche sauvage. Des tests d'infection de souris avec la souche $\Delta pknH$ ont indiqué une augmentation de la charge bactérienne dans les souris infectées par rapport aux souris infectées avec la souche sauvage (Papavinasasundaram *et al.*, 2005). De plus, lors de la vie intracellulaire de *M. tuberculosis*, la transcription de *pknH* est augmentée (Sharma *et al.*, 2006b). Ces résultats suggèrent que PknH jouerait un rôle primordial dans la survie de *M. tuberculosis* dans

le macrophage et dans la croissance bactérienne et, plus particulièrement, durant les phases chroniques d'infection.

Récemment, un gène homologue à *embR* a été identifié dans le génome de *M. tuberculosis* CDC1551. Ce gène, nommé *embR2*, n'est pas présent chez *M. tuberculosis* H37Rv. La protéine EmbR2 présente 55% d'identité de séquence primaire avec EmbR et comporte les mêmes domaines peptidiques. Bien que les résidus essentiels dans les domaines FHA soient similaires, EmbR2 n'est pas un substrat de PknH *in vitro*. En revanche, EmbR2 aurait un rôle d'inhibiteur, tant sur l'activité d'autophosphorylation de PknH que sur la phosphorylation de EmbR. Ces tests ont également permis de montrer que d'autres STPKs, telles que PknE et PknF, étaient capables de phosphoryler EmbR2 (Molle *et al.*, 2008).

Finalement, une approche bioinformatique a permis d'identifier 40 substrats potentiels de PknH. Deux d'entre eux, DacB1 et Rv0681, ont été analysés en détail. Rv0681 est un régulateur transcriptionnel possédant un motif HTH, alors que DacB1 est une protéine vraisemblablement impliquée dans la synthèse du peptidoglycane. Des tests de phosphorylation *in vitro* ont confirmé que ces deux protéines correspondent effectivement à des substrats de PknH bien que ne possédant pas de domaine FHA (Zheng *et al.*, 2007). Cette étude prouve que PknH est capable d'interagir et de phosphoryler différents substrats malgré l'absence du domaine caractéristique FHA identifié comme dans un autre substrat de PknH, EmbR.

1.3.3 Groupe 3: PknF/I/J

1.3.3.1 PknF

PknF est une protéine membranaire de 476 acides aminés (Koul *et al.*, 2001). Sur l'opéron contenant *pknF* se trouve également le gène *Rv1747*, codant un ABC transporteur (ATP Binding Cassette). Ces transporteurs sont des translocateurs chargés de la prise en charge et du transfert de molécules à travers la membrane plasmique via l'hydrolyse de molécules d'ATP. La phosphorylation étant un mécanisme connu de régulation de la stabilité et de l'activité des transporteurs ABC, une relation fonctionnelle entre PknF et *Rv1747* a été envisagée. *Rv1747* possède deux domaines FHA, suggérant que ce transporteur pourrait être régulé par phosphorylation. Des tests *in vitro* ont mis en évidence la phosphorylation de *Rv1747* par PknF, et des expériences de mutagenèse dirigée au niveau de résidus essentiels des domaines FHA de *Rv1747* ont permis de mettre en évidence une forte diminution de cette phosphorylation (Molle *et al.*, 2004).

Deux approches ont été menées afin d'appréhender le rôle physiologique de PknF, l'une par ARN anti-sens chez *M. tuberculosis*, l'autre par expression du gène de *M. tuberculosis* chez *M.*

smegmatis. Les bactéries exprimant un ARN antisens dirigé contre PknF présentent des septa anormaux, ainsi que des déformations au niveau des pôles, et montrent un temps de génération plus court que la normale (Deol *et al.*, 2005). De plus, les mutants anti-sens montrent une augmentation du transport de glucose par rapport à la souche sauvage, indiquant une régulation négative de PknF dans la régulation du trafic de glucose par le biais du transporteur Rv1747 (Deol *et al.*, 2005).

L'expression de PknF chez *M. smegmatis* entraîne, quant à elle, une croissance réduite ainsi que des changements sévères de morphologie. Ces observations suggèrent que PknF est impliquée à différents niveaux dans la régulation de la division cellulaire tels que la formation du septum et l'élongation (Deol *et al.*, 2005).

En parallèle, des expériences de délétion du gène *Rv1747* ont montré une croissance normale des mutants *in vitro*, alors que le mutant présentait une croissance réduite au niveau des poumons et de la rate de souris infectées. Ces résultats indiquent que la présence du transporteur Rv1747 est indispensable pour le développement de la bactérie *in vivo* (Curry *et al.*, 2005).

1.3.3.2 PknI

PknI est une protéine de 585 acides aminés dont le gène est en opéron avec des gènes impliqués dans la division cellulaire (*dacB* et *ftsY*). PknI a la particularité d'être la seule kinase mycobactérienne possédant une asparagine au niveau de son site de fixation de l'ATP au lieu de la lysine classiquement conservée chez les autres STPKs (Av-Gay & Everett, 2000). PknI serait exprimée au niveau du cytoplasme malgré la présence d'un domaine transmembranaire (Singh *et al.*, 2006).

Biochimiquement, PknI possède les caractéristiques d'une kinase de type STPK, notamment la capacité d'autophosphorylation, ainsi que celle de phosphoryler des substrats universels comme la MBP (Myelin Basic Protein) en présence de Mn^{2+} (Gopaldaswamy *et al.*, 2004). L'analyse du taux d'expression de *pknI* lors de l'infection de cellules THP-1 « macrophage like » avec *M. tuberculosis* a montré que *pknI* était initialement peu exprimé, et que l'expression diminuait encore au cours du temps (Singh *et al.*, 2006).

Plus récemment, des expériences de croissance dans le macrophage ainsi que chez la souris ont montré une hypervirulence des souches $\Delta pknI$. Par conséquent, PknI semble être impliquée dans la régulation de la multiplication de la bactérie lors de l'infection dans le macrophage et dans la persistance du bacille (Gopaldaswamy *et al.*, 2009).

1.3.3.3 PknJ

PknJ est une protéine de 61 kDa contenant une région transmembranaire. PknJ est présente uniquement chez *M. tuberculosis* et *M. bovis* (Narayan, 2007) mais, à l'heure actuelle, encore peu d'études ont été menées sur cette kinase.

1.3.4 Groupe 4: PknK et PknG

1.3.4.1 PknK

La combinaison des analyses bio-informatiques, de l'observation de son environnement génomique, ainsi que son architecture ont permis d'obtenir d'importantes informations préliminaires quant à ses fonctions. En effet, PknK possède un domaine de fixation à l'ADN au sein du domaine kinase (Av-Gay & Everett, 2000). D'autre part, le domaine C-terminal présente des homologies avec les régions régulatrices retrouvées notamment chez les régulateurs transcriptionnels de la famille LuxR (Av-Gay & Everett, 2000). Récemment, les travaux de Kumar et collaborateurs ont permis de caractériser biochimiquement PknK (Kumar *et al.*, 2009). Avec une masse moléculaire de 120 kDa, PknK correspond à la plus grande des STPKs de *M. tuberculosis*. De même que pour les autres STPKs mycobactériennes, on note la présence d'une lysine catalytique en position 55 et de deux thréonines essentielles en position 178 et 180 dans la boucle d'activation. PknK possède donc la capacité de s'activer par autophosphorylation et de phosphoryler un substrat générique tel que la MBP. En revanche, PknK ne possède pas de domaine transmembranaire, alors que l'analyse par fractionnement cellulaire indique que la protéine serait située au niveau de la membrane. Ceci pourrait s'expliquer du fait de la présence d'un domaine d'interaction de type PDZ (Post synaptic density protein, Drosophila disc large tumor suppressor A and Zonula occludens-1 protein), décrit comme possible domaine d'ancrage à la membrane.

Génétiquement, le gène *pknK* est adjacent au gène codant VirS, le régulateur transcriptionnel de l'opéron *mymA* (MYcobacterial Monooxygenase). Des tests de phosphorylation *in vitro* et *in vivo* ont ainsi mis en évidence que PknK était capable de phosphoryler VirS ainsi que quatre autres protéines faisant partie de l'opéron *mymA* (Mym, LipR, Rv3085, Rv3088). De manière intéressante, la phosphorylation de VirS par PknK entraîne non seulement une augmentation de l'affinité de VirS pour le promoteur de *mymA*, mais également une augmentation de la transcription de l'opéron. Par conséquent, PknK semble jouer un rôle primordial dans la régulation de la production de VirS et l'expression de l'opéron *mymA*. De plus, la délétion de *virS* et de *mym* entraîne des altérations phénotypiques de la paroi bactérienne. Il est donc fort probable que PknK soit, par le biais de VirS et de l'opéron *mymA*, impliquée dans le maintien de l'intégrité de la paroi mycobactérienne (Kumar *et al.*, 2009).

1.3.4.2 PknG

PknG est une protéine de 82 kDa pourvue d'une activité d'autophosphorylation et ne possédant pas de domaine transmembranaire. De même que pour PknK, cette particularité prédispose PknG à se localiser au niveau du cytoplasme. Cependant, l'analyse par Western blotting de différentes fractions cellulaires a montré que PknG se situait certes au niveau du cytoplasme, mais également de la membrane, ainsi que dans le milieu de culture (Cowley *et al.*, 2004).

La région N-terminale possède deux motifs CXXC. Ces motifs sont indispensables à la catalyse de réactions redox protégeant ainsi contre le stress oxydant via le contrôle de la formation/destruction des peroxydes (Tinsley *et al.*, 2004). Il a été également montré que cette portion N-terminale portait les sites de phosphorylation et était impliquée dans le recrutement des substrats de PknG (O'Hare *et al.*, 2008). La partie C-terminale, quant à elle, contient une région « tetratricopeptide (TRP)-like ». En effet, ce type de motif est retrouvé chez les eucaryotes (Brychzy *et al.*, 2003) et permettrait les interactions protéine/protéine comme précédemment étudié chez *B. subtilis* (Core & Perego, 2003).

Les souches de *M. tuberculosis* dont le gène *pknG* a été détruit montrent une croissance ralentie par rapport à la souche sauvage, démontrant ainsi que ce gène est essentiel pour une croissance optimale. Génétiquement, *pknG* est situé dans un opéron contenant *glnH*, gène codant une protéine impliquée dans le transport de la glutamine. Ainsi, les souches $\Delta pknG$ montrent chez *M. tuberculosis* une augmentation des concentrations cellulaires en glutamine et glutamate, suggérant que PknG serait impliquée dans la régulation métabolique de ces deux acides aminés. L'infection de souris par ces mêmes souches $\Delta pknG$ montre une survie prolongée des souris par rapport aux souris infectées par la souche sauvage. De plus, lors de l'infection dans le macrophage, les bacilles mutants présents dans les phagosomes sont rapidement transférés vers les lysosomes où ils seront détruits, alors que les bacilles sauvages sont capables de survivre dans les phagosomes non lysosomiaux (Walburger *et al.*, 2004). Il est intéressant de rappeler que l'inhibition ou le retardement de la fusion phagosome/lysosome est une des stratégies utilisées par les mycobactéries pour survivre chez l'hôte (Pieters, 2001 ; Nguyen & Pieters, 2005). Dans le but de comprendre si l'inhibition de la fusion phagosome/lysosome était liée à l'activité kinase de PknG, un mutant exprimant la forme inactive de la kinase a été généré, et cette protéine ainsi incapable de s'autophosphoryler a été surproduite chez *M. bovis* BCG. La surproduction de cette protéine mutante entraîne un transfert rapide des phagosomes aux lysosomes et donc la destruction intracellulaire des bactéries. Ces résultats tendent à montrer que l'activité kinase de PknG serait primordiale dans le contrôle du trafic phagosome/lysosome et, plus particulièrement, lors des mécanismes de persistance de *M. tuberculosis* dans le macrophage. PknG serait donc sécrétée et

phosphorylerait des substrats présents au niveau du cytoplasme du macrophage infecté (Walburger *et al.*, 2004). Cependant, il est important de noter que PknG ne possède pas de séquence signal classique permettant sa sécrétion à travers la membrane mycobactérienne. A l'heure actuelle, le mode de sécrétion de PknG ainsi que sa translocation dans le cytosol du macrophage restent à élucider.

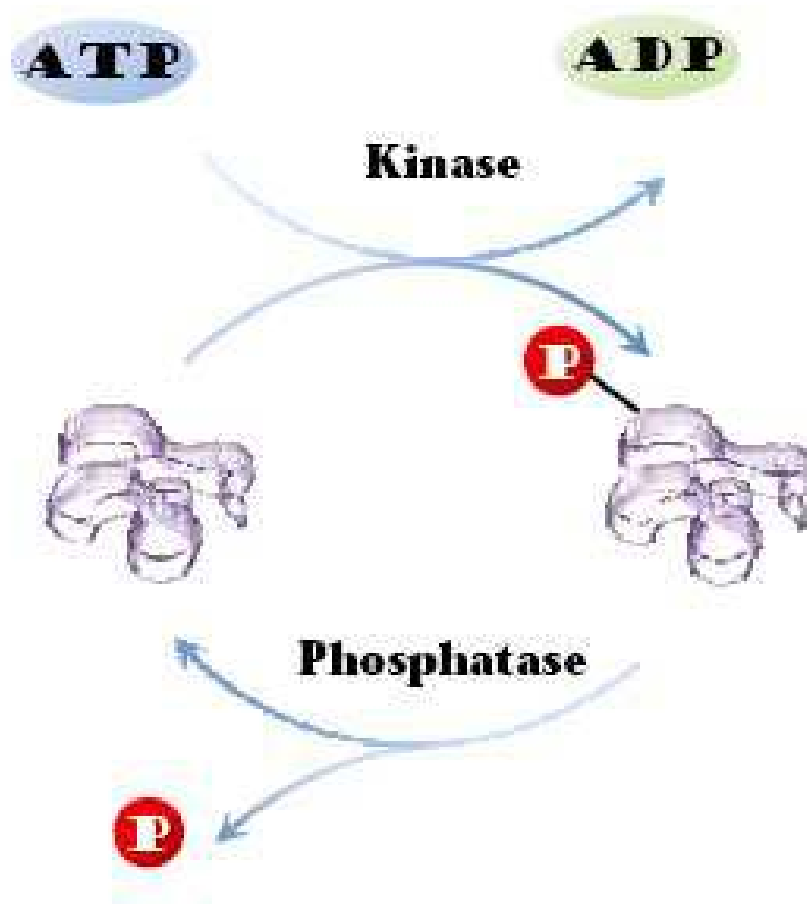


Figure n°1 : Schéma général des réactions de phosphorylation/déphosphorylation d'une protéine

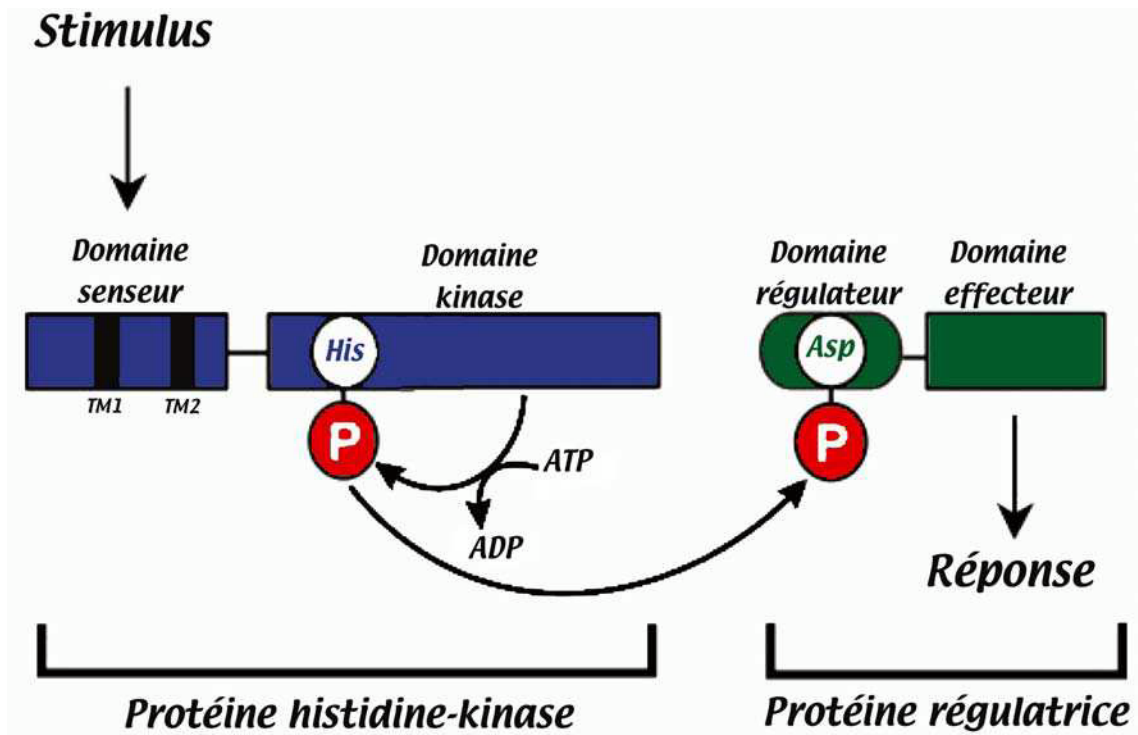


Figure n°2 : Schéma général de la transduction d'un signal par un système à deux composants

	I	II	
PrkC	-----MLIGK-----RISGRYQILRVIGGGMANVYLAEDIIL-DREVA IK ILRFD		
PrkD	ALKLLKLLFDRPLKNG-VILNHQYKIEECL GMGGYGLV YLCTDILA-QTP AKK EEKEKV		
Pkg2	-----MTTQPLATGDPRLRGLPYRLLGV LEGGMGK VYVGRDGS--GAP AAVK VLRPE		
Pkn2	-----MLAPDSL---VLDGRFRVLRPL SGGGMGEV YLGEQVSL-GRK VAIK VLVHHD		
PknB	-----MTTPS-----HLSDRYELGEI LFGG MSEVHLARDLRL-HRD VAIK VLRAD		
TGF-β	-----PIELDTLV GKGRFAE VYKAKLKQNTSE QFAVK IFPYE		
	. * * *	* * *	
	III	IV	
PrkC	YANDNEFIRRFRE EAQS ASSLDHNPVSIYDLGEEDD---IYYIVMEYVE GMTL KEYIT		
PrkD	-----VRFQ QEI KLLKNIHHPQIPGFIDEFIIDG---QAYYVMQFIE GENI EEELLF		
Pkg2	LAHDQHLAQRFVRE ADMAR AVTSKGVARVLGAQTEGG---RPWIAAEFL AGPTL DEAVR		
Pkn2	LHAQAGMAERFKRE EARLL SAVEHPAVRIVDFGSGD---HACLVMEF VEGESL YDVLT		
PknB	LARDPSFYLRFR EAQN AAALNHPAIVAVYDTGEAETPAGPLPYIVMEY VDGVT LRDIVH		
TGF-β	----EYASWKTE KEI FSDINLKHENILQFLTAEERKTEL GKQY WLITAF HAKNL QEYILT		
	. *	
	VI	VII	
PrkC	AN-GPLHPKEALNIMEQIVSAIAHA HQNQIVHRDIKPH NILIDHMG---NIK VTDFG IAT		
PrkD	FRKQPTELMALQLISQLEIIEY LHDLR LIF HSDIR TPNIIINDGR---LCL IDFGL AK		
Pkg2	TY-GPMDAPTVRALAAQLARTLHD IVAGL VHR DLK PANIVLTSTG---PRI IDFGI AR		
Pkn2	P--GPMPPGRALPLLQ LAEGL AA IHD KGI IHRDLK PENVFISKSARGE QARLLDFGI AR		
PknB	TE-GPMTPKRAIEVIADAC QALNF SHQNG IHRDV KPANIMISATN---AV KVMDFGI AR		
TGF-β	R--HVISWEDLRKLGSS LARGIAHL H KMP IVHR DLK SSN ILV KNDL---TC CLCDFGL SL		
	* . * * . * . .	. * * * .	
	VIII	IX	X
PrkC	ALSST---TITHTSNVLG SVHYLSPE OARGGLATK---K SDI YAL GIVL FELLTGR IPFD		
PrkD	QLT-----K--KNGTWLEELT----- PEEM EIKVRK---Q DDF DL GETL LLFLLYSQ-- YK		
Pkg2	FEHGL---TLTTT GQIP VT PGYGAPE QVLGQ R VGP---AS DV FS LGA VLAYAASGRRA FD		
Pkn2	LVEPDAASSVSQIGVVLGT PEYLSPE QAVGAKVDT---R SD LY SF GVLT YR VLSGR L P FD		
PknB	AIADSGN-SVTQ TAA VI GT AQYLS PE QARGDSVDA---R SD V Y SL GC VLYEVLT GE PP FT		
TGF-β	RLDPTLSVDDLANSGQ VTARYMAPE VLESRMNLEN F KQ TD V Y SMALV L WEMTSR CP PF G		
	**	*	.
	XI		
PrkC	GESAVSIALKHLQ AET PSAKRWN P SV P ---Q S VENI L KATA KDP PHRY E T A EDMEAD I KT		
PrkD	GK-----K--KNGTWLEELT-----LTKE VT LL LK RL L GI EE YQH---		
Pkg2	GAHVAAVQYEVVHG-TPDLSLV PP ELQ-----QLIG P CL SK DP AF RP AP LQ V AE A F AP		
Pkn2	GPLPRNFLSQHASA AP LP D RA AP TL S RYV GL LS V MR L E K D A SK R Q S A H E L A D A L A A		
PknB	GDSFVSVAYQHVRED P IP S AR H E G LS---AD L DA V V L K A L A K N P E NR Y Q T A E M R AD L V R		
TGF-β	SKVKDNVLRDRGR PEI PS F W L N H Q G I Q ---M V C E T L T E C W D H D P E A R L T A Q C V A E R F S E L		
	.	*	.

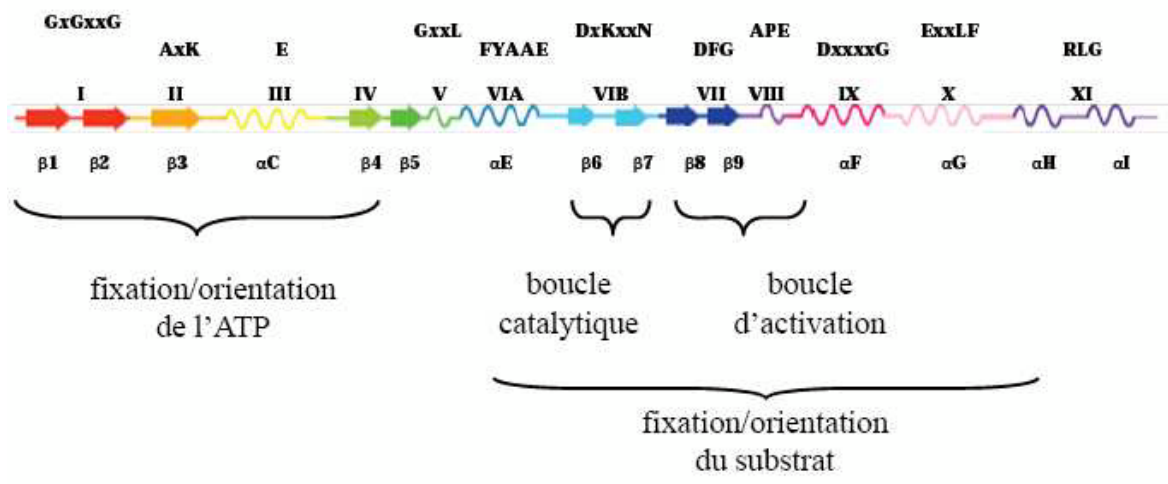


Figure n°3 : Alignement de séquences de différentes protéine-kinases de type Hanks
PkrC de *B. subtilis* ; PrkD de *B. subtilis*; Pkg2 de *S. granaticolor*; Pkn2 de *M. xanthus*; PknB de *M. tuberculosis* et TGF-β de *Homo sapiens*.

(D'après Madec *et al.*, 2002)

Histidine protéine-kinases

CheA	PEKRLAAGKNSVGNL (14) VTDDGAGLNR (41) SGRGVGMDVV
EnvZ	PLSIKRAVANMVVNA(22) VEDDGP GIAP (19) SGTGLGLAIV
NtrB	PDQIEQVLLNIVRNA (35) VEDNGP GIPP (15) GGTGLGLSIA
KinA	QNQLKQVFINLIKNA (24) VKDEGEG IPE (15) NGTGLGLTVT
KinB	ATKLLQAVINLMKNG (25) ITDNGV GMTD (15) NGTGLGLTVT
KinC	ENQLKQVFINIIKNG (24) VKDEGNG MPQ (15) KGTGLGLPIC

Sérine protéine-kinases

SpoIIAB	LTEIKTVVSEAVTNA (27) IRDEGLGITD (15) ERSGMGFTIM
RsbW	IEDLKIAVSEACTNA (27) VADEGDSF-D (20) SEGGLGLYLM
RsbT	QARITTAISELARNI (25) AEDQGPGIPD (12) GGLGAGLPGV

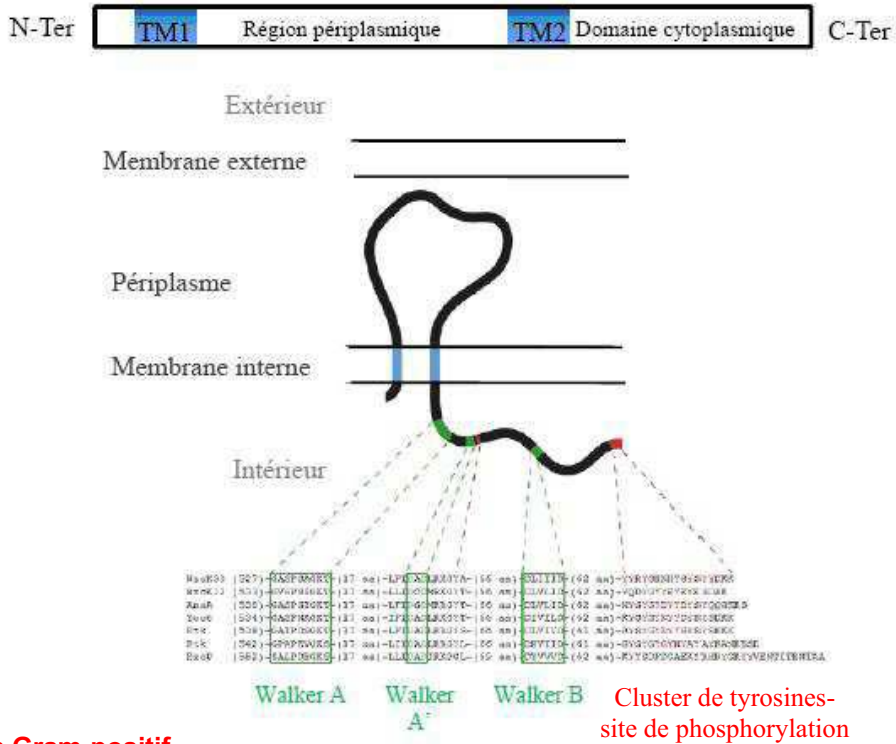
Figure n° 4 : Alignement de trois Ser/Thr protéine-kinases dites à motif histidine-kinase SpoIIAB et RsbW, RsbT de *B. subtilis*, ainsi que de six His protéine-kinases bactériennes.

Les résidus conservés dans les motifs N, G1 et G2 sont en rouge. Ils sont importants pour la fixation de l'ATP, mais aussi pour l'activité kinase.

(D'après Kang *et al.*, 1998)

Bactérie à Gram-négatif

Protéobactérie



Bactérie à Gram-positif

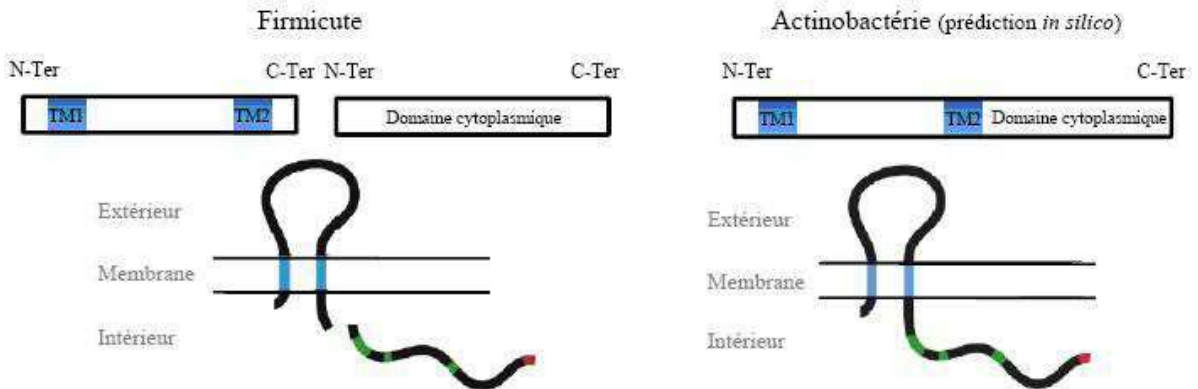


Figure n°5 : Organisation et domaines conservés chez les Tyr kinases bactériennes (BY kinases)

(Adapté à partir de Grangeasse *et al.*, 2007)

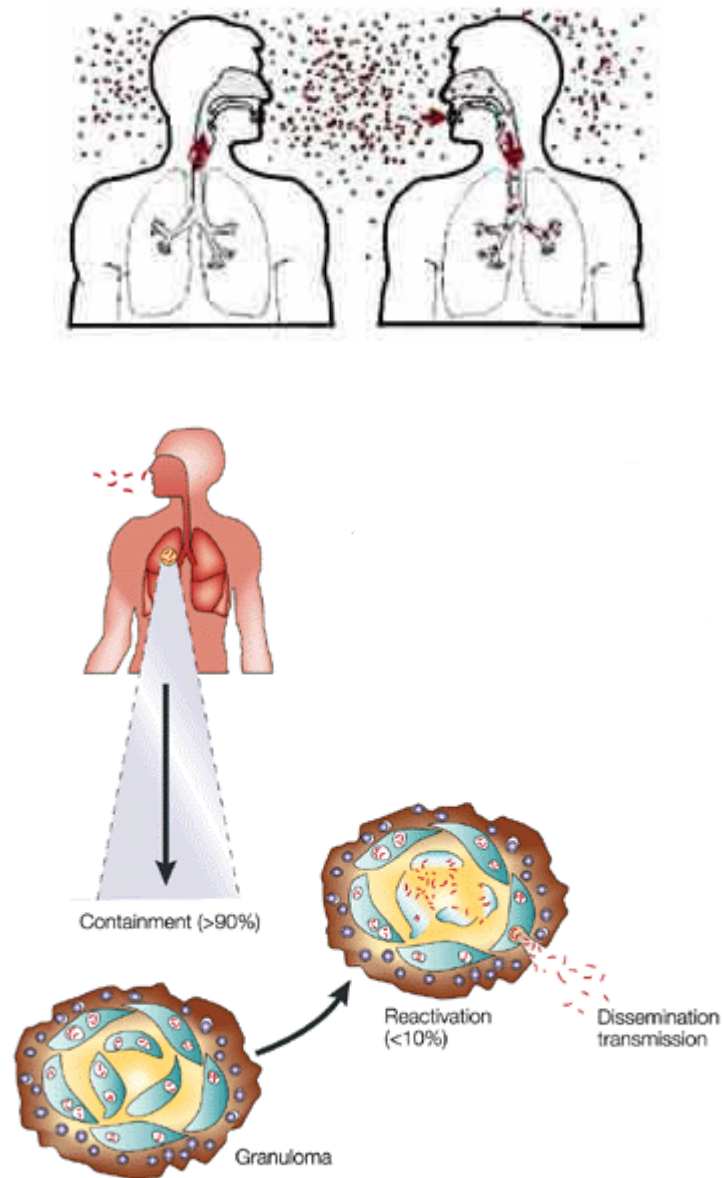


Figure n°6 : Schéma de la transmission et de l'infection par *Mycobacterium tuberculosis* des macrophages alvéolaires et formation d'un granulome.

Le Bacille de Koch est un agent pathogène strictement humain dont la forme pulmonaire assure principalement la transmission inter-humaine. La bactérie provoque des lésions pulmonaires très riches en germes et se retrouve vaporisée dans des gouttelettes - gouttelettes de Flügge- de 1 à 5 μm de diamètre lors de l'expectoration d'un patient atteint de la maladie. La transmission se fait d'homme à homme par voie aérienne par inhalation des gouttelettes, suffisamment petites pour atteindre les alvéoles pulmonaires. Au niveau des alvéoles, les bactéries sont phagocytées par les macrophages alvéolaires. Le recrutement d'autres cellules immunitaires conduit à la formation d'un granulome. Lors de la réactivation de la bactérie, les granulomes sont détruits, libérant et provoquant la dissémination des bacilles.

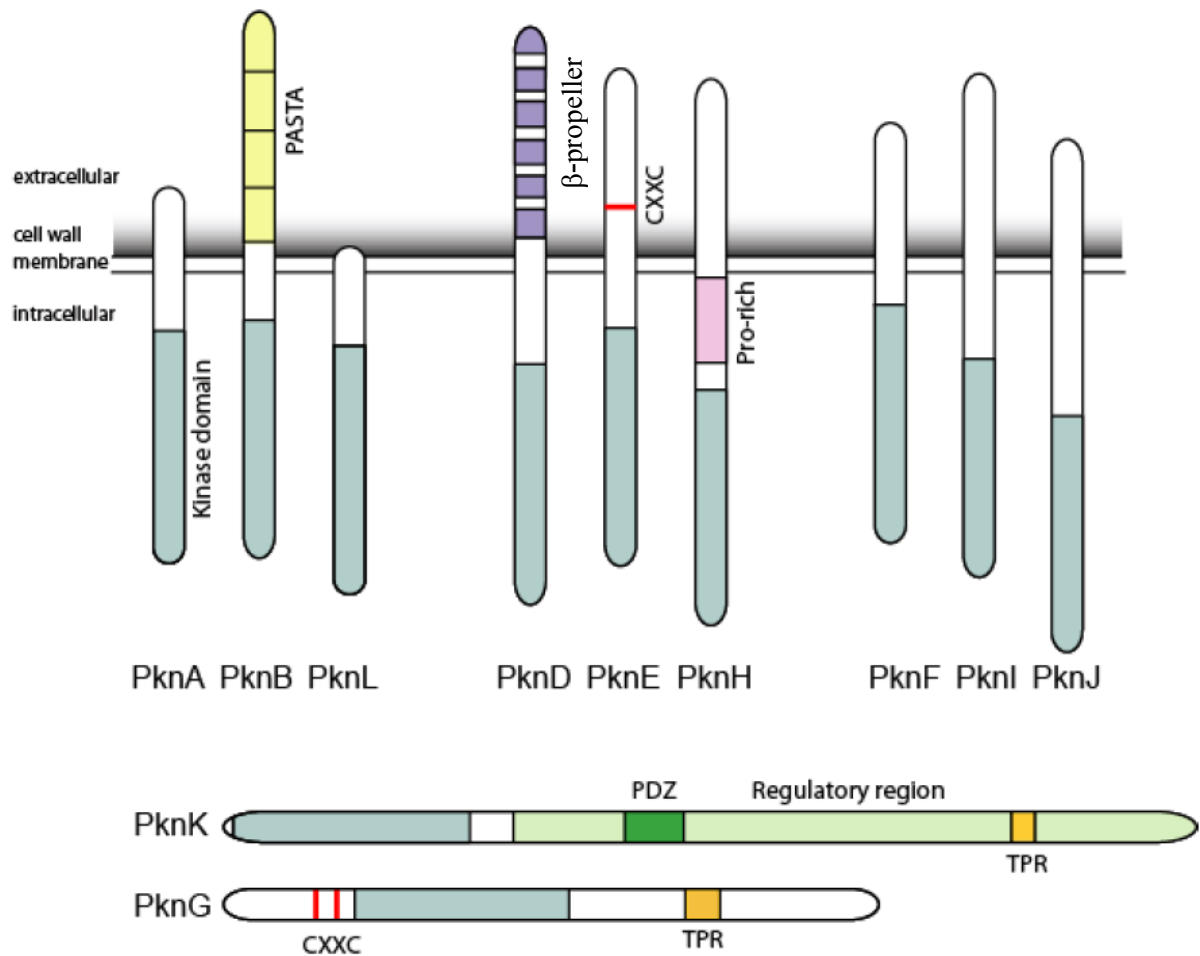


Figure n°7 : Schéma représentatif des onze STPKs de *Mycobacterium tuberculosis*.

Parmi les onze STPKs de *M. tuberculosis*, neuf présentent des domaines transmembranaires, les deux autres (PknK et PknG) étant cytoplasmiques. Différents motifs caractéristiques sont représentés le long des séquences des kinases.

Kinase domain : domaine kinase

PASTA : penicillin A binding and Ser/Thr kinase attached

β -propeller : hélice β

CXXC : motif thioredoxine

Pro-Rich : motif riche en proline

PDZ : motif d'ancrage à la membrane

TPR : motif « Tetratricopeptide repeat »

(Adapté de la thèse de Nicole Scherr, 2008)

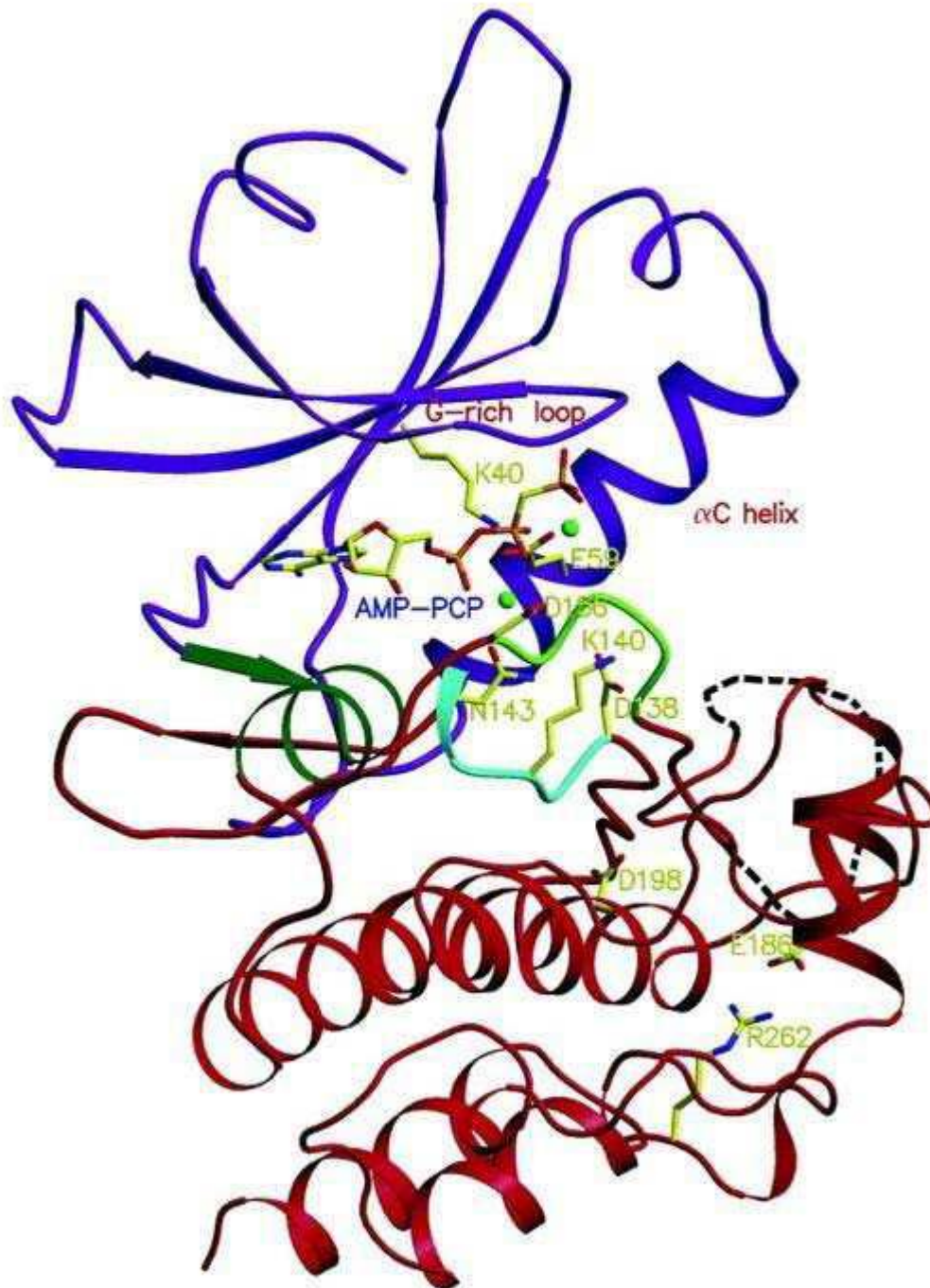


Figure n°8 : Structure tridimensionnelle du domaine catalytique de PknB

Sur cette figure, est présenté l'aspect bi-lobé du domaine kinase de PknB avec le sous-domaine N-terminal en bleu et le sous-domaine C-terminal en rouge. Les résidus essentiels sont annotés en jaune et la boucle catalytique est représentée en bleu cyan. L'hélice C α sous-domaine N-terminal est également visible.

(D'après Ortiz-Lombardia *et al.*, 2003)

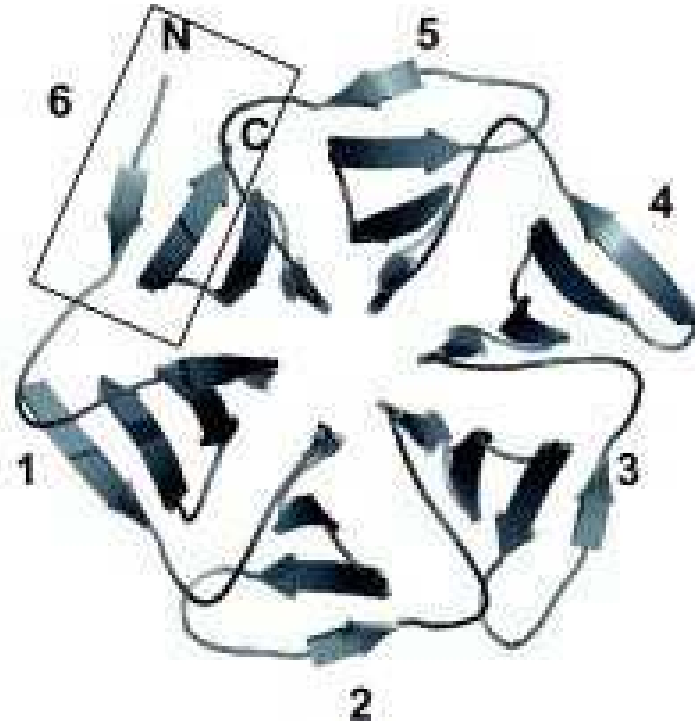


Figure n°9 : Structure en hélice β du domaine extracellulaire de PknD

Le domaine extracellulaire de PknD forme une hélice β à six lames. Chaque lame est composée de quatre feuillets β reliés entre eux par des boucles. Les lames de l'hélice sont organisées autour d'un pore central.

(D'après Good *et al.*, 2004)

Chapitre 2

Résultats

Dans ce manuscrit, le *premier article* présente la caractérisation biochimique de la Ser/Thr protéine-kinase PknL de *M. tuberculosis*, ainsi que l'étude de son substrat Rv2175c. Le *deuxième article* porte sur la caractérisation structurale de Rv2175c obtenue par RMN, ainsi que sur le rôle de la phosphorylation dans la régulation de son activité de fixation à l'ADN. Le *troisième article* concerne la construction d'un vecteur plasmidique spécifique à la surproduction de protéines compatible avec les conditions des tests de phosphorylation *in vitro*. Le *quatrième article* détaille l'identification de la protéine chaperonne GroEL1 de *M. tuberculosis* en tant que nouveau substrat de différentes STPKs mycobactériennes, ainsi que la caractérisation de ses sites de phosphorylation.

Le constat majeur de ces différentes études réside dans le fait que différentes STPKs sont capables de phosphoryler différents substrats possédant différentes fonctions.

En annexe, sont présentés, dans deux articles, les résultats des études que nous avons menées en collaboration avec l'équipe du Prof. José Gil (Université de Léon, Espagne) sur l'identification et la caractérisation des STPKs de *Corynebacterium glutamicum*, ainsi que sur l'identification d'un substrat spécifique, MurC, impliqué dans la synthèse du peptidoglycane. L'un des résultats notables est que deux des quatre kinases présentes chez *C. glutamicum*, PknA et PknB, ainsi que le substrat MurC, sont impliqués dans la régulation et la production du peptidoglycane. Par conséquent, les kinases corynébactériennes semblent jouer un rôle prépondérant dans la régulation de la division cellulaire chez cet organisme modèle.

Enfin, l'ensemble des résultats est discuté dans le chapitre *Discussion et Conclusion*.

Article I - The *Mycobacterium tuberculosis* serine/threonine kinase PknL phosphorylates Rv2175c: mass spectrometric profiling of the activation loop phosphorylation sites and their role in the recruitment of Rv2175c

Canova MJ, Veyron-Churlet R, Zanella-Cleon I, Cohen-Gonsaud M, Cozzone AJ, Becchi M, Kremer L, and Molle V.

Proteomics. 2008, 8:521-33

Article II - The *Mycobacterium tuberculosis* Ser/Thr kinase substrate Rv2175c is a DNA-binding protein regulated by phosphorylation

Cohen-Gonsaud M, Barthe P, Canova MJ, Stagier-Simon C, Kremer L, Roumestand C, and Molle V.

Journal of Biological Chemistry 2009, In press

Article III - pETPhos: a customized expression vector designed for further characterization of Ser/Thr/Tyr protein kinases and their substrates

Canova MJ, Kremer L, and Molle V.

Plasmid 2008, 60:149-53

Article IV - The *Mycobacterium tuberculosis* GroEL1 chaperone is a substrate of Ser/Thr Protein Kinases

Canova MJ, Kremer L, and Molle V.

Journal of Bacteriology 2009, 191: 2876-2883

Article Annexe I - From the characterization of the four serine/threonine protein kinases (PknA/B/G/L) of *Corynebacterium glutamicum* toward the role of PknA and PknB in cell division

Fiuza M, Canova MJ, Zanella-Cléon I, Becchi M, Cozzzone AJ, Mateos LM, Kremer L, Gil JA, and Molle V

Journal of Biological Chemistry 2008, 283:18099-112

Article Annexe II - The MurC ligase essential for peptidoglycan biosynthesis is regulated by the serine/threonine protein kinase PknA in *Corynebacterium glutamicum*

Fiuza M, Canova MJ, Patin D, Letek M, Zanella-Cléon I, Becchi M, Mateos LM, Mengin-Lecreulx D, Molle V, and Gil JA

Journal of Biological Chemistry 2008, 283:36553-63

Article I

The *Mycobacterium tuberculosis* serine/threonine kinase PknL phosphorylates Rv2175c: mass spectrometric profiling of the activation loop phosphorylation sites and their role in the recruitment of Rv2175c

Canova MJ, Veyron-Churlet R, Zanella-Cleon I, Cohen-Gonsaud M, Cozzone AJ, Becchi M, Kremer L, and Molle V

Proteomics. 2008, 8:521-33.

Présentation de l'article I :

La phosphorylation des protéines est une des voies majeures de signalisation par laquelle les signaux extracellulaires sont transférés, intégrés et traduits en réponse cellulaire.

Chez les procaryotes, ces voies de signalisation font intervenir, entre autres, la famille des "eukaryotic-like" Ser/Thr Protein-Kinases (STPKs).

Chez *M. tuberculosis*, le séquençage intégral du génome a permis de révéler la présence de onze STPKs. Des études récentes ont permis de mettre en évidence que les STPKs étaient au centre d'un grand nombre de systèmes de régulation, certaines étant notamment impliquées dans la pathogénie de la bactérie. Par conséquent, leur caractérisation représente un enjeu primordial pour identifier de nouvelles cibles thérapeutiques.

Nos travaux ont porté sur l'identification et la caractérisation biochimique de PknL, une des onze STPKs détectées *in silico* lors du séquençage du génome de *M. tuberculosis*. Nous avons, tout d'abord, confirmé que PknL possédait une activité Ser/Thr kinase. Pour ce faire, des expériences de phosphorylation *in vitro*, puis de mutagenèse dirigée, nous ont permis de confirmer l'activité d'autophosphorylation de PknL. De plus, les tests de phosphorylation réalisés avec différents mutants au niveau de différents résidus d'acides aminés conservés ont permis de mettre en évidence le caractère essentiel du résidu catalytique lysine 49, ainsi que le rôle primordial des résidus phosphorylés, thréonines 173 et 175, présents au niveau de la boucle d'activation de PknL. Afin de compléter l'étude biochimique de PknL, une analyse de spectrométrie de masse a permis d'identifier cinq sites de phosphorylation sur la kinase.

Durant cette étude, nous avons également mis en évidence que PknL était capable de recruter et de phosphoryler Rv2175c, une protéine dont le gène est adjacent à celui codant la kinase dans le génome. Des tests de phosphorylation *in vitro* de Rv2175c avec les différents mutants de PknL ont montré que la phosphorylation du résidu Thr173 de PknL était indispensable à la phosphorylation optimale de Rv2175c par PknL.

De plus, nous avons confirmé *via* la surproduction et purification à partir de cultures de *Mycobacterium smegmatis* que la protéine Rv2175c était phosphorylée *in vivo* et présente sous plusieurs isoformes caractéristiques de son état de phosphorylation.

RESEARCH ARTICLE

The *Mycobacterium tuberculosis* serine/threonine kinase PknL phosphorylates Rv2175c: Mass spectrometric profiling of the activation loop phosphorylation sites and their role in the recruitment of Rv2175c

Marc J. Canova¹, Romain Veyron-Churlet², Isabelle Zanella-Cleon¹, Martin Cohen-Gonsaud^{3, 4, 5}, Alain J. Cozzone¹, Michel Becchi¹, Laurent Kremer^{2, 6} and Virginie Molle¹

¹ Institut de Biologie et Chimie des Protéines, CNRS UMR 5086, Université Lyon 1, IFR128 BioSciences, Lyon-Gerland, Lyon, France

² Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, Université de Montpellier II et I, CNRS UMR 5235, Montpellier, France

³ CNRS UMR 5048, Centre de Biochimie Structurale, Montpellier, France

⁴ INSERM U554, Montpellier, France

⁵ Université Montpellier I et II, Montpellier, France

⁶ INSERM, DIMNP, Montpellier, France

Although *Mycobacterium tuberculosis* (*M. tb*) comprises 11 serine/threonine protein kinases, the mechanisms of regulation of these kinases and the nature of their endogenous substrates remain largely unknown. Herein, we characterized the *M. tb* kinase PknL by demonstrating that it expresses autophosphorylation activity and phosphorylates Rv2175c. On-target dephosphorylation/MALDI-TOF for identification of phosphorylated peptides was used in combination with LC-ESI/MS/MS for localization of phosphorylation sites. By doing so, five phosphorylated threonine residues were identified in PknL. Among them, we showed that the activation loop phosphorylated residues Thr173 and Thr175 were essential for the autophosphorylation activity of PknL. Phosphorylation of the activation loop Thr173 residue is also required for optimal PknL-mediated phosphorylation of Rv2175c. Together, our results indicate that phosphorylation of the PknL activation loop Thr residues not only controls PknL kinase activity but is also required for recruitment and phosphorylation of its substrate. Rv2175c was found to be phosphorylated when overexpressed and purified from *Mycobacterium smegmatis* as 2-DE indicated the presence of different phosphorylated isoforms. Given the presence of the *dcw* gene cluster in the close vicinity of the *pknL/Rv2175c* locus, and its conservation in all mycobacterial species, we propose that PknL/Rv2175c may represent a functional pair in the regulation of mycobacterial cell division and cell envelope biosynthesis.

Received: May 8, 2007
Revised: September 5, 2007
Accepted: October 14, 2007

**Keywords:**

Mycobacterium tuberculosis / Phosphorylation / PknL / Mass spectrometry

Correspondence: Dr. Virginie Molle, Institut de Biologie et Chimie des Protéines, 7 passage du Vercors, 69367 Lyon Cedex 07, France

E-mail: vmolle@ibcp.fr

Fax: +33-4-72-72-26-01

Abbreviations: HTH, helix-turn-helix; *M. tb*, *Mycobacterium tuberculosis*; STPKs, serine/threonine protein kinases; TEV, tobacco etch virus

1 Introduction

Tuberculosis is the world's most serious infectious disease, killing over two million people annually [1]. The causative bacterial agent, *Mycobacterium tuberculosis* (*M. tb*), has a distinctive life cycle spanning different environments and developmental stages. Especially, the ability of *M. tb* to enter macrophages and to avoid intracellular killing is believed to be pivotal to its virulence. Moreover, *M. tb* can lie dormant for

years to re-emerge when the immune system wanes through old age, AIDS (acquired immuno-deficiency syndrome) or malnutrition. Therefore, a better understanding of the bacterial response and adaptation to these modifications in host cell environment, relies on the study of regulatory proteins involved in mycobacterial signal transduction pathways.

Modification of proteins by phosphorylation on a limited number of amino acid residues is a general mechanism used to control protein activities in both eukaryotic and prokaryotic cells. Protein phosphorylation is a principal mechanism by which extracellular signals are translated into cellular responses [2]. Three main superfamilies of protein kinases were identified, one including 'eukaryotic-like' serine/threonine protein kinases (STPK) [3–5], one corresponding to the newly characterized prokaryotic class of tyrosine kinases [6] and one to the well-known family of bacterial kinases, the sensor histidine-kinases, which are key enzymes of the so-called 'two-component systems' [7]. In bacteria, the presence of several STPKs suggests a central role of protein phosphorylation in regulating various biological functions, ranging from environmental adaptative responses to bacterial pathogenicity. Thus, regulatory devices involving STPKs and phosphatases, represent an emerging theme in prokaryotic signalling cascades.

Analysis of the genome sequence of *M. tb* predicted the presence of 11 different STPKs [8, 9]. Nine of these appear to be transmembrane proteins, with a putative extracellular signal sensor domain (except PknL), and an intracellular kinase domain. Some of them have been investigated for their physiological roles [10–20]. PknG and PknH have been implicated in the pathogenesis and survival of *M. tb* within the host [11, 21]. Consequently, discovering the mechanism of regulation of bacterial Ser/Thr phosphosignalling systems has been the focus of many recent studies. However, only a few substrates have been documented to date [17, 22–34].

The focus of this work is the *M. tb* PknL kinase, which had not been characterized previously. This kinase consists of 399 amino acids, with a putative single transmembrane-spanning helix located between amino acids V₃₇₀ and G₃₉₂, dividing the protein in an N-terminal cytoplasmic domain and a C-terminal membrane domain. A recent bioinformatic study revealed that *pknL* of *M. tb* is conserved in all mycobacterial species sequenced [24], including *M. leprae*, despite the extensive gene decay in this bacillus [35]. This strongly suggests that PknL could regulate essential physiological functions. The analysis of the amino acid sequence of the protein indicates that the N-terminal region contains all of the 11 consensus catalytic subdomains of the Hanks-type kinase which are required for a functional kinase activity [36]. Interestingly, *pknL* is associated with the ~30 kb *dcw* (division cell wall) gene cluster involved in cell envelope biosynthesis and cell division [24], raising the possibility that PknL might participate in the regulation of this gene cluster. Further, *pknL* (*Rv2176*) is adjacent to the *Rv2175c*, a gene encoding a putative DNA-binding transcriptional reg-

ulator [8, 9], and several studies have reported phosphorylation of proteins encoded by genes that were adjacent to their cognate kinases on the mycobacterial chromosome [17, 22, 26, 28]. As a first step in deciphering the potential regulation of the *dcw* gene cluster by *Rv2175c* and PknL, we have studied the link between PknL and *Rv2175c*. Here, we demonstrate that PknL is a STPK able to recruit and phosphorylate *Rv2175c*, and we identify the residues of the PknL activation loop that appear to be critical for autophosphorylation activity as well as recruitment/phosphorylation of the substrate.

2 Materials and methods

2.1 Bacterial strains and growth conditions

Strains used for cloning and expression of recombinant proteins were *Escherichia coli* DH5 α (Clontech Laboratories) and *E. coli* BL21(DE3)B834 (Novagen), respectively. All strains were grown and maintained in LB medium at 37°C. When required, media were supplemented with 100 μ g/mL ampicillin, and/or 25 μ g/mL kanamycin. *M. smegmatis* mc²155 was grown on Middlebrook 7H11 agar plates containing oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco). Liquid cultures of *M. smegmatis* mc²155 were grown in Sauton's medium, supplemented with 25 μ g/mL kanamycin when necessary.

2.2 Cloning, expression and purification of the recombinant PknL protein

Expression and purification of recombinant GST-tagged PknL₁₋₃₆₉ was performed as described earlier [25]. Recombinant *E. coli* BL21(DE3)B834 strains harbouring the kinase-expressing construct pGEX(M)-*pknL*₁₋₃₆₉ were used to inoculate 100 mL of LB medium supplemented with ampicillin and were incubated at room temperature with shaking until A₆₀₀ reached 0.5. IPTG was then added at a final concentration of 1 mM, and growth was continued for an additional 2 h at room temperature, with shaking. Purifications of the GST-tagged recombinant proteins were performed as reported previously [25] except that binding and wash buffers were supplemented with glycerol (10% final, w/v) in order to maintain the protein in an active form.

2.3 Construction and purification of *Rv2175c* recombinant protein

The *Rv2175c* gene was amplified by PCR using *M. tb* H37Rv genomic DNA as a template and the following primers: #403, 5'-TAT ATA TCG TT CAT atg CCT GGC CGC GCA CCA GGC TCT-3' (containing a *Nde*I restriction site underlined) and, #404, 5'-TAT GGA TCC TCA ATA CGC CAT AGC CTG GGC CCG-3' (containing a *Bam*HI restriction site underlined). This 441-bp amplified product was diges-

ted by *NdeI* and *BamHI*, and ligated into the variant of pET15b (Novagen) that includes the replacement of the thrombin site coding sequence with a tobacco etch virus (TEV) protease site [37]. *E. coli* BL21(DE3)B834 cells transformed with this construct were used for expression and purification of His-tagged Rv2175c as previously described [25]. Finally, the purified His-tagged Rv2175c was treated with TEV protease according to the manufacturer's instructions (Invitrogen).

2.4 Cloning and purification of PknL₁₋₃₆₉ mutant proteins

Site-directed mutagenesis was carried out based on PCR amplification. The strategy consisted in creating substitutions in PknL₁₋₃₆₉ proteins. A first set of PCR amplifications was generated using pGEX(M)-*pknL*₁₋₃₆₉ as a template along with the primer pair, #196 (5'-TAT GGA TCC GTG GTC GAA GCT GGC ACG-3') and #300 (5'-GA ATC CAT CAC CAT CAG CGC GAC-3'), #196 and #307 (5'-GCC GGT AGA CGC GAT TGA AGC-3'), #196 and #308 (5'-GAT GAC GCC GGC AGA CGT GAT-3'), #196 and #309 (5'-GAT GAC GCC GGC AGA CGC GAT TGA AGC-3'), #196 and #425 (5'-GGT AGA CGT GAT AGC GGC GGC GAC-3'), #196 and #429 (5'-GAT GAC GCC GGT AGC CGT GAT TGA AGC-3') were used to generate *pknL*₁₋₃₆₉-K48M, *pknL*₁₋₃₆₉-T173A, *pknL*₁₋₃₆₉-T175A, *pknL*₁₋₃₆₉-T173A/T175A, *pknL*₁₋₃₆₉-S171A, *pknL*₁₋₃₆₉-S174A, respectively. These primers were then used in a second round of PCR amplification in combination with primer #197 (5'-TAT AAG CTT TTA TCG ACG GGC GTG CTG TCG-3') to generate the entire 1107 bp *pknL*₁₋₃₆₉ gene. These different DNA fragments, containing the appropriate sites at both ends, were restricted by *BamHI* and *HindIII* (sites underlined) and ligated into the pGEX(M) [17] previously digested with the same enzymes. The different GST-tagged recombinants fusion proteins were overexpressed and purified as reported earlier [25].

2.5 In vitro kinase assay

In vitro phosphorylation of about 500 ng of PknL₁₋₃₆₉ protein was carried out for 30 min at 37°C in a reaction mixture (20 µL) containing buffer P (25 mM Tris-HCl, pH 7.0; 1 mM DTT; 5 mM MgCl₂; 1 mM EDTA) with 200 µCi/mL [γ -³²P]ATP. Phosphorylation of Rv2175c by PknL₁₋₃₆₉ was performed with 3 µg of Rv2175c in 20 µL of buffer P with 200 µCi/mL [γ -³²P]ATP and 500 ng of PknL₁₋₃₆₉ for 30 min at 37°C. The reaction was stopped by addition of an equal volume of 2 × sample buffer and the mixture was heated at 100°C for 5 min. After electrophoresis, gels were soaked in 16% TCA for 10 min at 90°C, and dried. Radioactive proteins were visualized by autoradiography using direct exposure to films.

In vitro phosphorylation for MS analysis was performed as described above except that [γ -³²P]ATP was replaced by 5 mM nonradioactive ATP.

2.6 Analysis of the phosphoamino acid content of proteins

Protein samples (PknL₁₋₃₆₉ or Rv2175c) were separated by 1-D gel electrophoresis and electroblotted onto an Immobilon PVDF membrane. Phosphorylated proteins bound to the membrane fraction were detected by autoradiography. The ³²P-labelled protein bands were excised from the Immobilon blot and hydrolysed in 6 M HCl for 1 h at 110°C. The acid-stable phosphoamino acids thus liberated were separated by electrophoresis in the first dimension at pH 1.9 (800 V·h) in 7.8% acetic acid/2.5% formic acid, followed by ascending chromatography in the second dimension in 2-methyl-1-propanol/formic acid/water (8:3:4). After migration, radioactive molecules were detected by autoradiography. Authentic phosphoserine, phosphothreonine and phosphotyrosine were run in parallel and visualized by staining with ninhydrin.

2.7 Overexpression and purification of Rv2175c in *M. smegmatis*

Standard PCR strategies were used to amplify the *M. tb* H37Rv *Rv2175c* gene, using the following primers: #318, 5'-TAA TAG CTC ATA TGC CTG GCC GCG CAC CA-3' (containing a *NdeI* site underlined) and #319, 5'-TAT AAG CTT ATA CGC CAT AGC CTG GGC-3' (containing a *HindIII* site underlined). The PCR product was cut with *NdeI* and *HindIII*, enabling direct cloning into the pVV16 expression vector cut with *NdeI/HindIII* [38]. This plasmid is a derivative of pMV261 [39], containing both a kanamycin and a hygromycin resistance cassette, harbouring the *hsp60* promoter as well as a His-tag for expression of C-terminal His-tagged fusion proteins. The resulting expression vectors, named pVV16-*Rv2175c* was used to transform *M. smegmatis* mc²155. Purification of soluble His-tagged Rv2175c from recombinant *M. smegmatis* was performed on Ni-NTA agarose beads as described previously [25].

2.8 Mass spectrometry analysis (MALDI-TOF and LC-ESI/MS/MS)

Purified GST-tagged fusion proteins of PknL₁₋₃₆₉ were subjected to *in vitro* phosphorylation with nonradioactive ATP prior to resolving on SDS-PAGE. In-gel digestion was performed as described by Shevchenko *et al.* [40] with minor modifications. Reduction was performed with 60 µL of 10 mM DTT in 50 mM NH₄HCO₃ for 40 min at 56°C. Alkylation was performed with 60 µL of 55 mM iodoacetamide in 50 mM NH₄HCO₃ for 30 min at room temperature in dark. The gel pieces were successively dried, rehydrated and redried using 300 µL of CH₃CN, 200 µL of 50 mM NH₄HCO₃ and 300 µL of CH₃CN, respectively. For proteolytic digestion, the gel was treated by 30–40 µL of a trypsin (sequence grade, Promega, Madison, WI, USA) for 5 h at 37°C. The resulting proteolytic peptides were extracted from

the gel by centrifugation and the supernatant fraction was recovered. A second extraction step was performed using 30–40 μL $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{HCOOH}$ (60:36:4; v/v/v) mixture for 30 min and, finally, all extracts were pooled and used for MALDI-TOF MS. For LC-ESI/MS/MS experiments, an aliquot of the extract was dried in a vacuum concentrator and resuspended in 0.1% TFA.

A Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems, Courtaboeuf, France) was used for peptide mass mapping in positive ion reflector mode and positive ion linear mode. Spectra were acquired in the 700–5000 Da mass range. A 1 mg/200 μL solution of CHCA (LaserBioLab, Sophia-Antipolis, France) in $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ mixture (50:50) containing 0.1% TFA was used as matrix.

For on-target dephosphorylation alkaline phosphatase was from calf intestine (1 U/ μL , Roche, Meylan, France) was used. Tryptic peptide digest solution (1 μL) was spotted on the MALDI target and allowed to dry, due to the acid concentration of the sample. Dialysed phosphatase solution (1–2 μL) was added. The MALDI target was then rapidly placed in a Petri dish on a wet tissue, and incubated at 37°C (incubator) for 1.5 h. The target was removed from the Petri dish and the solution, phosphatase and peptide digest, was allowed to dry. Finally, 1 μL of matrix solution (CHCA) was added, mixed and dried.

NanoLC/nanospray/MS/MS (LC-ESI/MS/MS) experiments were performed on a Q-STAR XL instrument (QqTOF) (Applied Biosystems) equipped with a nanospray source using a distal coated silica-tip emitter (FS 150-20-10-D-20, New Objective, USA) set at 2300 V. By means of information dependant acquisition mode (IDA), peptide ions within a m/z 400–2000 survey scan mass range were analysed for subsequent fragmentation (three precursors). Double to quadruple charged ions exceeding a threshold of ten counts were selected for MS/MS analyses. MS/MS spectra were acquired in the m/z 50–2000 range with 30 s dynamic exclusion time. The collision energy was automatically set by the software (Analyst 1.1) and was related to the charge of the precursor ion. The MS and MS/MS data were recalibrated using internal reference ions from a trypsin autolysis peptide at m/z 842.510 $[\text{M} + \text{H}]^+$ and m/z 421.759 $[\text{M} + 2\text{H}]^{2+}$.

Screening for phosphorylated peptides was achieved by the paragon method from the Protein Pilot® database searching software (version 1.0, Applied Biosystems). The LC part of the analytical system consisted of a LC-Packings nano-LC (Dionex, Voisins Le Bretonneux, France). Chromatographic separation of peptides was obtained in a C_{18} PepMap microprecolum (5 μm ; 100 \AA ; 300 $\mu\text{m} \times 5$ mm; Dionex) and a C_{18} PepMap nano-column (3 μm ; 100 \AA ; 75 $\mu\text{m} \times 150$ mm; Dionex). After injection (1 μL injection volume, pick-up mode, in a 20 μL injection loop), samples were adsorbed and desalted on the precolum with a $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{TFA}$ (98:2:0.05; v/v/v) solvent mixture for 3 min at 25 $\mu\text{L}/\text{min}$ flow rate. The peptide separation was developed using a linear 60 min gradient from 0 to 60% B, where solvent A was 0.1% HCOOH in

$\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (95:5) and solvent B was 0.1% HCOOH in $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (20:80) at approximately 200 nL/min flow rate.

2.9 Western blot analysis and 2-DE

To separate and detect the different phosphorylated isoforms of Rv2175c, 5 μg of protein purified from *M. smegmatis* pVV16-Rv2175c were electrophoresed on immobilized 7 cm pH 6.3–8.3 gradient strips on a Protean IEF Cell (Biorad) in the first dimension and on a 15% SDS-PAGE in the second dimension. The proteins were either stained in CBB or immunodetected with INDIA HisProbe-HRP (SuperSignal West HisProbe Kit, Pierce Chemicals), a nickel-activated derivative of HRP, able to directly detect the blotted recombinant His-tagged Rv2175c fusion protein. The SuperSignal® kit (Pierce) and BioMax film (Kodak) were used for protein visualization.

3 Results and discussion

3.1 Expression and purification of the cytosolic domain of PknL

The *pknL* gene encodes a 399-aminoacid protein, with a calculated molecular mass of 43 kDa and an estimated pI of 6.19. Analysis of the aminoacid sequence revealed the presence of all the essential amino acids and sequence subdomains characterising the Hanks' family of eukaryotic-like protein kinases [36]. These include the central core of the catalytic loop, consisting of subdomain VI, and the invariant residue Lys48 in the consensus motif within subdomain II, which is usually involved in the phosphotransfer reaction and required for the autophosphorylating activity of eukaryotic STPKs (Fig. 1A) [36, 41]. The hydropathy profile of PknL revealed a single, putative transmembrane spanning α -helix, located between amino acids V₃₇₀ and G₃₉₂, constituting a membrane anchor (Fig. 1A) [42]. The extracellular portion of STPKs normally consists of one or more sensor domains that, when bound to their ligand lead to a change in the intracellular conformation, which activates a signalling cascade. In some cases, this extracellular domain has been shown to possess PASTA domains (penicillin-binding-protein and serine/threonine kinase-associated domain) [43]. In the *M. tb* STPK PknB for instance, all of the extracellular portion is predicted to consist of four PASTA domains, which strongly suggests that it is a signal-binding sensor domain [43]. Surprisingly, PknL is unique among the *M. tb* STPKs in lacking a substantial extracellular domain [9, 24], suggesting that this enzyme may respond to a different type of ligand than the other SPTKs. To allow detailed biochemical studies, we focused our attention on the N-terminal cytosolic domain of PknL corresponding to residues Met₁ to Met₃₆₉, and comprising the catalytic domain (Fig. 1A). The truncated *pknL* gene lacking the hydrophobic domain

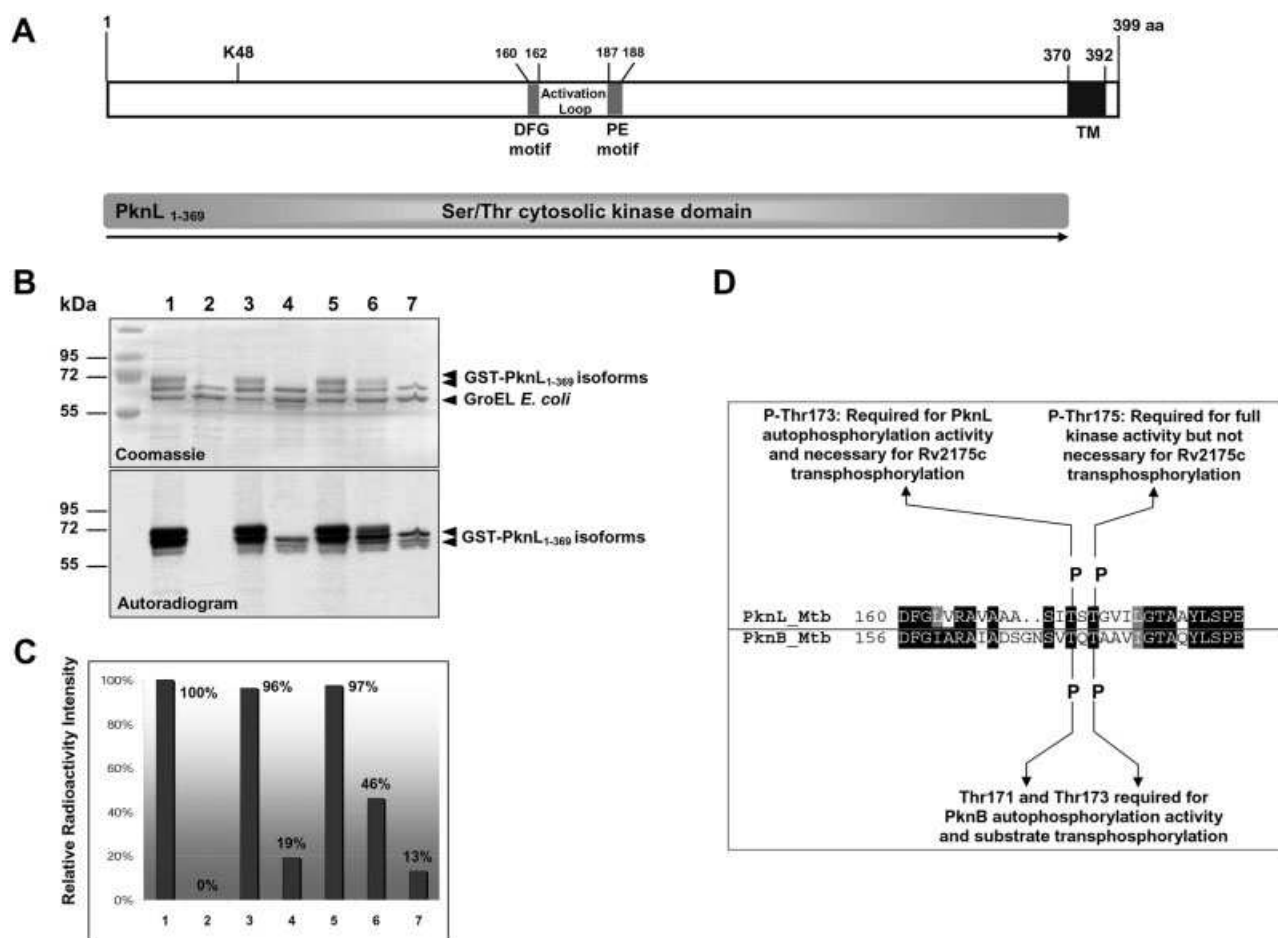


Figure 1. Schematic representation of PknL and *in vitro* autophosphorylation of the cytosolic kinase domain. (A) *M. tb* *pknL* encodes a predicted transmembrane protein with a cytosolic N-terminal domain characterized by the typical activation loop delimited by the DFG and PE motifs of the STPK family connecting a single transmembrane helix. (B) *In vitro* phosphorylation of the different derivatives of PknL₁₋₃₆₉. All proteins were overproduced in *E. coli* and purified as GST fusions. The following proteins were incubated with [γ -³²P]ATP: PknL₁₋₃₆₉ (lane 1), PknL₁₋₃₆₉-K48M (lane 2), PknL₁₋₃₆₉-S171A (lane 3), PknL₁₋₃₆₉-T173A (lane 4), PknL₁₋₃₆₉-S174A (lane 5), PknL₁₋₃₆₉-T175A (lane 6), PknL₁₋₃₆₉-T173A/T175A (lane 7). Proteins were separated by SDS-PAGE and stained with CBB (upper panel), and radioactive bands were revealed by autoradiography (lower panel). (C) Radioactivity was measured with a Molecular Dynamics Typhoon phosphoimager and plotted. The activity of the wild-type enzyme was arbitrarily put at 100%. (D) Amino acid sequence comparison of the activation loops of the *M. tb* STPKs PknL and PknB. The key autophosphorylated threonine residues are indicated by a 'P'.

(*pknL*₁₋₃₆₉) was synthesized by PCR amplification using genomic DNA from *M. tb* H37Rv. The amplified product was cloned into plasmid pGEX(M) to yield pGEX(M)-*pknL*₁₋₃₆₉ and used to transform *E. coli*. The recombinant GST-tagged PknL₁₋₃₆₉ fusion protein was purified with glutathione-sepharose 4B matrix. Analysis of the GST-chimeric protein by SDS-PAGE revealed that the wild-type enzyme was expressed in a soluble form, and migrated with the predicted molecular mass of approximately 70 kDa, but also showed the presence of extra bands (Fig. 1B, upper panel). The upper bands analysed by MS confirmed the identity of the PknL₁₋₃₆₉ GST-chimeric protein, and analysis of the electrophoretic migration profile showed that PknL₁₋₃₆₉ migrated as a diffuse band with a higher molecular mass than the predicted one

(Fig. 1B, lane 1, upper panel). This aberrant migration property has been observed previously for other *M. tb* kinases, such as PknD, PknE and PknH [10, 16, 17], as well as for Pkn9 and Pkn6, two *Mycobacterium xanthus* STPKs [44, 45]. It was demonstrated that this was due to the different phosphorylated isoforms. Further analysis by MS revealed that the lower band corresponded to the GroEL protein of *E. coli* that usually copurified with the GST-tagged PknL₁₋₃₆₉, presumably allowing to maintain the kinase in a stable soluble form (Fig. 1B, upper panel). As expected, the two upper bands likely corresponding to the GST-tagged PknL₁₋₃₆₉ isoforms, but not the lower band corresponding to GroEL, reacted positively to anti-GST antibodies, thus confirming the identity of these proteins (data not shown).

3.2 Protein kinase activity of PknL₁₋₃₆₉

To demonstrate that the cytosolic domain of PknL displays STPK activity, PknL₁₋₃₆₉ was incubated with [γ -³²P]ATP as a phosphate donor. Wild-type PknL₁₋₃₆₉ incorporated radioactive phosphate from [γ -³²P]ATP, giving rise to a radioactive signal at around 70 kDa, corresponding to the expected size of the protein isoforms, indicating that PknL undergoes autophosphorylation (Fig. 1B, lane 1, lower panel). To exclude the possibility of exogenous contamination that could explain labelling of PknL with [γ -³²P]ATP, Lys48, present in subdomain II of PknL₁₋₃₆₉, was mutated to Met. Purified PknL₁₋₃₆₉-K48M, was incubated in the presence of [γ -³²P]ATP, and as expected, no signal could be detected (Fig. 1B, lane 2, lower panel). This confirms that PknL₁₋₃₆₉ expresses autophosphorylation activity and that Lys48 of subdomain II is essential for catalysing the phosphorylation reaction, in agreement with previous reports [36, 46].

We next determined the nature of the amino acid residues phosphorylated in PknL₁₋₃₆₉. Following labelling with [γ -³²P]ATP, PknL₁₋₃₆₉ was isolated by SDS-PAGE and subjected to acid hydrolysis. Only acid-resistant phosphoamino acids can be detected since a number of other phosphorylated residues, such as phosphohistidine, phosphoarginine and phosphoaspartate, are known to be labile when subjected to acidic treatment. The corresponding autoradiogram shows that PknL₁₋₃₆₉ was mainly phosphorylated on threonine residues, and to a lesser extent on serines (Fig. 2A). Altogether, these data indicate that the N-terminal cytosolic domain of PknL has intrinsic autophosphorylation activity *in vitro*.

3.3 Contribution of the activation loop Thr173 and Thr175 residues in the autophosphorylation activity of PknL

Various mechanisms of eukaryotic protein kinase regulation have been described. The transition between active and inactive forms may occur *via* control of access to the catalytic site and/or the substrate-binding site. A large number of these regulatory mechanisms involve phosphorylation/dephosphorylation through an autocatalytic mechanism or by action of other intervening kinases and phosphatases. The activation loop is a major control element of an active/inactive conformational switch in numerous kinases [47], and the conformation of the activation loop often depends on the phosphorylation state [48]. Based on structural studies, it is thought that the activation loop controls both the accessibility to the catalytic site and the binding of the substrate. Recently, Zheng *et al.* [49] demonstrated that proteins sharing a similar amino acid sequence with the autophosphorylation sites of the PknH activation loop were indeed able to undergo *in vitro* phosphorylation by the PknH kinase, thus confirming the major role of this motif in the kinase-substrate recognition mechanism.

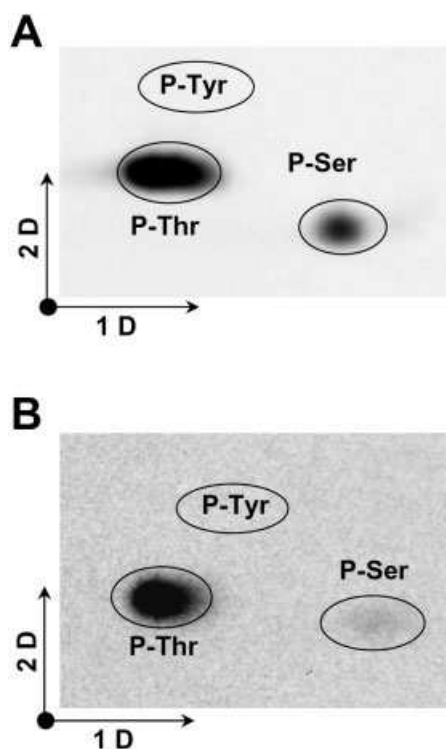


Figure 2. Phosphoamino acid content of PknL₁₋₃₆₉ and Rv2175c. Proteins labelled with [γ -³²P]ATP were analysed by SDS-PAGE, electroblotted onto an immobilon PVDF membrane, excised and hydrolysed in acid. The phosphoamino acids thus liberated were separated by electrophoresis in the first dimension (1-D) and ascending chromatography in the second dimension (2-D). After migration, radioactive molecules were detected by autoradiography. Authentic phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) were run in parallel as internal standard controls, and visualized by ninhydrin staining. (A) Phosphoamino acid content of PknL₁₋₃₆₉. (B) Phosphoamino acid content of Rv2175c phosphorylated by PknL₁₋₃₆₉ following cleavage with the TEV protease.

In an elegant study, Boitel *et al.* [50] have established Thr171 and Thr173 as essential phosphothreonine residues in PknB kinase activity. Substitutions of Thr171 or Thr173 by Ala decreased the activity by 15 or 20 times compared to the wild-type enzyme, whereas the double mutant was 300-fold less active. This confirmed that double phosphorylation of the activation loop is required for full kinase activity and demonstrated the involvement of both phosphothreonines in the regulatory mechanism of PknB activity. When mapped on the crystal structure of *M. tb* PknB [51, 52], the activation loop was defined as the region spanning from the conserved DFG to the PE motifs, corresponding to residues 160–188 in PknL (Fig. 1A). Interestingly, this segment in PknL includes Thr173 and Thr175, corresponding to Thr171 and Thr173 in PknB (Fig. 1D), two of the four phosphorylated residues of PknB, described as phosphorylation sites in the activation loop of PknB [52]. The presence of such residues in PknL supports the con-

cept that phosphorylation of the activation loop plays a regulatory role as recently described for other mycobacterial STPKs [32, 50].

In order to determine a possible link between Thr173 and Thr175 and the autophosphorylation activity of PknL₁₋₃₆₉, the two residues were individually substituted by Ala to generate PknL₁₋₃₆₉-T173A and PknL₁₋₃₆₉-T175A, respectively. In addition, a double mutant was also created and designated PknL₁₋₃₆₉-T173A/T175A (Fig. 1B, lanes 4, 6, 7, upper panel). The electrophoretic migration profile showed that PknL₁₋₃₆₉-T173A migrated as a nondiffuse band compared to the wild-type migration pattern (Fig. 1B, lane 4, upper panel), whereas PknL₁₋₃₆₉-T175A migrated with a diffuse band, albeit less intense than compared to the wild-type protein (Fig. 1B, lane 6, upper panel). We reasoned that, because these proteins displayed different electrophoretic mobility properties, they must differ in their intrinsic phosphorylation states.

This hypothesis was confirmed by labelling the mutant proteins *in vitro* with [γ -³²P]ATP. PknL₁₋₃₆₉-T173A gave rise to a radioactive signal much lower than the wild-type enzyme (Fig. 1B, lower panel, lane 4). Quantification of the signal intensity indicated an autophosphorylation activity of 19% with respect to the activity of the wild-type protein, which was arbitrarily placed at 100% (Fig. 1C, lane 4). This result strongly argues that Thr173 is a key phosphorylation site of the activation loop, necessary for PknL₁₋₃₆₉ autophosphorylation activity. The signal intensity generated by PknL₁₋₃₆₉-T175A was intermediate between that of the wild-type protein and the Thr173 mutant (Fig. 1B, lower panel, lane 6), representing 46% of residual activity with respect to the wild-type protein activity (Fig. 1C, lane 6). Therefore, this result confirms the requirement of Thr175 for optimal PknL₁₋₃₆₉ autophosphorylation activity, although this residue appears less important than Thr173. When both Thr residues were mutated, the intensity of the signal was similar to the one of the single Thr173 mutant (Fig. 1B, lower panel, lane 7 and Fig. 1C, lane 7). Together, our results confirm that double phosphorylation of the activation loop residues

Thr173 and Thr175 is necessary for full kinase activity and unambiguously demonstrate the role of both phosphothreonines.

Due to the presence of phosphorylated serines (Fig. 2A) in PknL, and because two serine residues, Ser171 and Ser174, are present in the close vicinity of Thr173 and Thr175 (Fig. 1D), we investigated whether they may be phosphorylated residues of the activation loop. Recombinant PknL₁₋₃₆₉-Ser171A and PknL₁₋₃₆₉-Ser174A were purified and labelled *in vitro* with [γ -³²P]ATP. Both proteins gave rise to radioactive signals similar to the one obtained for the wild-type protein (Fig. 1B, lower panel, lanes 3 and 5), which was further confirmed following densitometry analysis (Fig. 1C). These results rule out the possibility of Ser171 and Ser174 as being phosphorylated residues in PknL₁₋₃₆₉.

3.4 Identification of the phosphorylated amino acid residues in PknL₁₋₃₆₉ and PknL₁₋₃₆₉ mutants

To identify the phosphoresidues in the wild-type PknL₁₋₃₆₉ protein as well as in the PknL₁₋₃₆₉ activation loop mutants, an MS approach was used. This technique has proven to be a method of choice for characterizing PTMs [53–57]. On-target dephosphorylation/MALDI-TOF was used for the identification of phosphorylated peptides and LC-ESI/MS/MS for localization of phosphorylation sites, in the PknL₁₋₃₆₉, PknL₁₋₃₆₉-T173A, PknL₁₋₃₆₉-T175A, PknL₁₋₃₆₉-T173A/T175A, PknL₁₋₃₆₉-S171A and PknL₁₋₃₆₉-S174A proteins. The tryptic peptides from the in-gel digestion of the different PknL₁₋₃₆₉ proteins were identified by MALDI-TOF MS in reflectron mode and by LC-ESI/MS/MS, which lead to 75% of sequence coverage. Therefore, one cannot exclude the possibility of additional phosphorylated serine or threonine residues not covered in this analysis. Phosphorylated peptides were identified by MALDI-TOF MS in linear mode in combination with on-target alkaline phosphatase treatment [57]. Mass decrements of 80 Da observed by differential MALDI peptide mass mapping lead to the identification of phosphorylated peptides. Phosphorylated amino acid residues were assigned by pep-

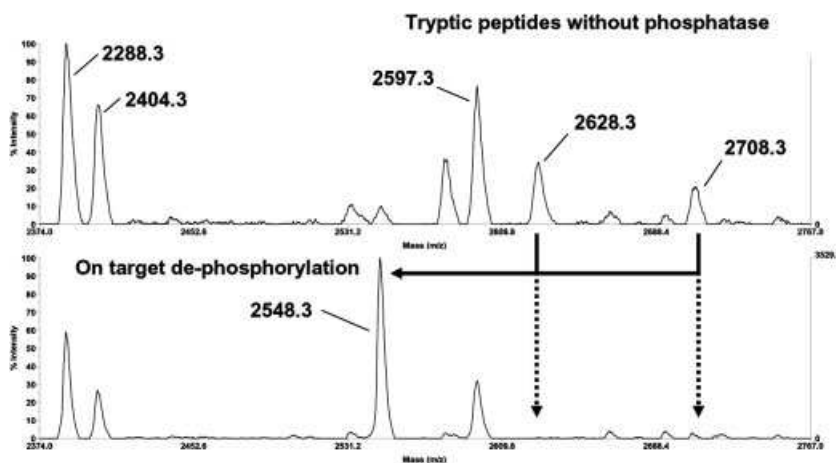


Figure 3. MALDI-TOF spectrum of PknL₁₋₃₆₉ before and after on-target dephosphorylation. Linear MALDI-TOF mass spectrum of tryptic digest peptides prior to (upper panel) or following to (lower panel) alkaline phosphatase treatment. Bold arrows indicate loss of 1 × 80 Da and 2 × 80 Da from GST-PknL₁₋₃₆₉ peptide [166–191] with a decrease in *m/z* 2628.3 and 2708.3 to *m/z* 2548.3, respectively.

Table 1. Phosphorylation status of GST-tagged recombinant protein kinases PknL₁₋₃₆₉ and mutants as determined by MS (see also Supporting Information)

Phosphorylated tryptic sequence	Number of detected phosphate groups		Phosphorylated residue ^{a)}
	MALDI-MS	LC-ESI/MS/MS	
PknL₁₋₃₆₉-WT			
[25–35] IASGGTSTVYR	nf	1	T32
[54–65] YAGDEQFLTRFR	1	1	T62
[166–191] AVAAASITSTGVILGTAAYLSPEQVR	1	1	T173 or 175
	2	2	T173 and T175
[308–324] ITQOQQLGAKPVVHPTR	nf	1	T323
PknL₁₋₃₆₉-T173A			
[25–35] IASGGTSTVYR	0	0	
[54–65] YAGDEQFLTRFR	0	0	
[166–191] AVAAASIA <u>S</u> TGVILGTAAYLSPEQVR	1	1	T175
[308–324] ITQOQQLGAKPVVHPTR	nf	1	T323
PknL₁₋₃₆₉-T175A			
[25–35] IASGGTSTVYR	nf	1	T32
[54–65] YAGDEQFLTRFR	1	1	T62
[166–191] AVAAASIT <u>S</u> AGVILGTAAYLSPEQVR	1	1	T173
[308–324] ITQOQQLGAKPVVHPTR	nf	1	T323
PknL₁₋₃₆₉-T173A/T175A			
[25–35] IASGGTSTVYR	0	0	
[54–65] YAGDEQFLTRFR	0	0	
[166–191] AVAAASIA <u>S</u> AGVILGTAAYLSPEQVR	0	0	
[308–324] ITQOQQLGAKPVVHPTR	nf	1	T323
PknL₁₋₃₆₉-S171A			
[25–35] IASGGTSTVYR	nf	1	T32
[54–65] YAGDEQFLTRFR	1	1	T62
[166–191] AVAAAA <u>I</u> TSTGVILGTAAYLSPEQVR	1	1	T173 or T175
	2	2	T173 and T175
[308–324] ITQOQQLGAKPVVHPTR	nf	1	T323
PknL₁₋₃₆₉-S174A			
[25–35] IASGGTSTVYR	nf	1	T32
[54–65] YAGDEQFLTRFR	1	1	T62
[166–191] AVAAASIT <u>A</u> TGVILGTAAYLSPEQVR	1	1	T173 or T175
	2	2	T173 and T175
[308–324] ITQOQQLGAKPVVHPTR	nf	1	T323

nf, phosphorylated peptide not found; underlined, mutated residue.

a) Phosphorylation sites were determined from the analysis of LC-ESI/MS/MS spectra.

tide fragmentation in MS/MS: γ and b daughter ions containing one phosphoserine or phosphothreonine were associated with a neutral loss of phosphoric acid ($-H_3PO_4$, *i.e.* -98 Da).

As detailed in Table 1, analysis of tryptic digests of PknL₁₋₃₆₉ allowed detection of four phosphorylated peptides. Among these, the [166–191] peptide exhibited multiphosphorylation, therefore leading to the identification of five phosphorylation sites in total in wild-type PknL₁₋₃₆₉ (Table 1). These sites correspond to Thr32, Thr62, Thr323, in

addition to Thr173 and Thr175. Figure 3 shows the example of the phosphorylated peptide [166–191] from wild-type PknL₁₋₃₆₉ corresponding to the diphosphorylated isoform of the kinase. The mass spectrometric results tells us that, (i) two isoforms of the kinase constitute the active pool of PknL₁₋₃₆₉, consistent with their SDS-PAGE migration profiles (Fig. 1B upper panel), (ii) these isoforms correspond to the mono- or diphosphorylated state of the activation loop phosphothreonines Thr173 and Thr175, (iii) the ratio between the mono- or diphosphorylated state of the activation

loop peptide was close to one, indicating that these threonines are reaching an equilibrium between the two isoforms.

However, the phosphorylation status of the T173A mutant is mainly diphosphorylated, with two sites corresponding to Thr323 and Thr175 (Table 1). As Thr175 appeared to be a phospho-site in the wild-type protein, one can assume that the Thr175 can be phosphorylated solely in the activation loop of this mutant, but this would not restore wild-type activity, and would not play a functional role, as similarly described for PknB by Boitel *et al.* [50]. The Thr173 mutant appeared impaired in its autophosphorylation activity as Thr32 and Thr62 identified as phosphorylated sites in the wild-type kinase could not be phosphorylated (Table 1). Therefore, the Thr173 phosphorylated site seems to be essential for autophosphorylation activity.

Four sites of phosphorylation compared to the five detected in the wild-type kinase, and the two detected in the Thr173 mutant, were identified in the Thr175 mutant. One can assume that the Thr173 can be phosphorylated solely in the activation loop of the Thr175 mutant, but this would only restore partial activity of the kinase as shown in Fig. 1C, even if Thr32, Thr62 and Thr323 are being phosphorylated. Thus, Thr175 does not seem to be necessary for autophosphorylation but appears to be essential to reach full kinase activity as compared to the wild-type (Fig. 1C).

Finally, the phosphorylated residues of recombinant PknL₁₋₃₆₉-Ser171A and PknL₁₋₃₆₉-Ser174A were similar to the ones obtained for the wild-type protein (Table 1). These results comfort our *in vitro* phosphorylation assay that ruled out the possibility of Ser171 and Ser174 as being phosphorylated residues in PknL₁₋₃₆₉ (Fig. 1B, lower panel, lanes 3 and 5).

In summary, both Thr173 and Thr175 of the activation loop appeared to be essential for the kinase activity of PknL₁₋₃₆₉ as confirmed with the double mutant Thr173/Thr175 being only weakly phosphorylated on one site, Thr323 (Table 1). Thus, MS coupled to *in vitro* kinase assays demonstrated unambiguously the involvement of both phosphothreonines in PknL₁₋₃₆₉ activity. Overall, the number of phosphorylation sites identified for PknL₁₋₃₆₉ and each of its mutants by mass spectrometric analyses allowed us to confirm explicitly our *in vitro* kinase assay in terms of phosphorylation intensity and migration profile (Table 1, Figs. 1B and C).

PknB, the most widely studied *M. tb* STPK, also possesses the two characteristic threonines in its activation loop (Thr171 and Thr173), while two other *M. tb* STPKs PknE and PknH only harbour a single Thr residue, equivalent to Thr171 in PknB, or Thr173 in PknL [9]. In PknB not only both residues were found to be phosphorylated, but also PknB autophosphorylation activity was dramatically reduced in both the single and the double mutants [50]. In PknH, Thr170 of the activation loop was shown to be required for autophosphorylation activation, and recruitment of its FHA-containing substrate, EmbR [17]. Thus, PknL possesses a PknB-like activation loop, in which both Thr173 and Thr175

are required for full kinase activity, with Thr173 being more critical for its activation.

These observations are consistent with a recent phylogenetic analysis showing that all mycobacterial STPKs are clustered into five clades and that both PknL and PknB belong to the clade I [24].

3.5 Rv2175c is a substrate of PknL

Because Rv2175c is adjacent to *pknL* and has been proposed to participate in regulating the neighbouring genes in the *dcw* cluster [24], we investigated whether Rv2175c is a substrate for PknL. Rv2175c is a 146-aminoacid protein, with a calculated molecular mass of 16 kDa and an estimated *pI* of 7.8. Rv2175c shows weak similarity to transcriptional regulatory proteins harbouring a DNA-binding domain (residues 32–52) with a helix-turn-helix (HTH) motif (Fig. 4A).

Rv2175c was expressed, purified from *E. coli* as a His-tagged protein, and cleaved with the TEV protease in order to remove the N-terminal His-tag. Analysis of the purified recombinant protein by SDS-PAGE revealed that the protein was expressed in a soluble form with the expected molecular weight of 16 kDa (Fig. 4B, upper panel). The ability of PknL₁₋₃₆₉ to phosphorylate Rv2175c was examined in an *in vitro* phosphorylation assay in the presence of [γ -³²P]ATP. As shown in Fig. 4B (lane 1, lower panel), PknL₁₋₃₆₉ could phosphorylate Rv2175c, whereas Rv2175c alone was unable to incorporate [γ -³²P] (Fig. 4B, lane 8), thus confirming that Rv2175c is a substrate of PknL₁₋₃₆₉.

Next, to determine the nature of the phosphorylated aminoacids, the phosphoamino acid content of PknL-phosphorylated Rv2175c was investigated. The protein was labelled with [γ -³²P]ATP *in vitro* as described above, purified by SDS-PAGE and subjected to acid hydrolysis. Figure 2B showed that Rv2175c was preferentially phosphorylated on threonine. Thus, PknL₁₋₃₆₉ is a STPK capable of autophosphorylation on both serine and threonine residues (Fig. 2A) and able to transphosphorylate Rv2175c on threonines (Fig. 2B).

3.6 Phosphorylation of Rv2175c is dependent on the activation loop Thr173 residue

The fact that PknL/Rv2175c represents a novel kinase/substrate pair in *M. tb* prompted us to analyse the contribution of different key residues of PknL in the transphosphorylation mechanism between the kinase and its substrate. This was achieved by incubating recombinant Rv2175c with PknL₁₋₃₆₉-K48M, PknL₁₋₃₆₉-S171A, PknL₁₋₃₆₉-T173A, PknL₁₋₃₆₉-S174A, PknL₁₋₃₆₉-T175A or PknL₁₋₃₆₉-T173A/T175A. PknL₁₋₃₆₉-K48M was unable to phosphorylate Rv2175c, indicating that phosphorylation of PknL₁₋₃₆₉ is a prerequisite to the transphosphorylation reaction (Fig. 4B, lower panel, lane 2). More importantly, whereas the T173A mutation completely abolished the transphosphorylation

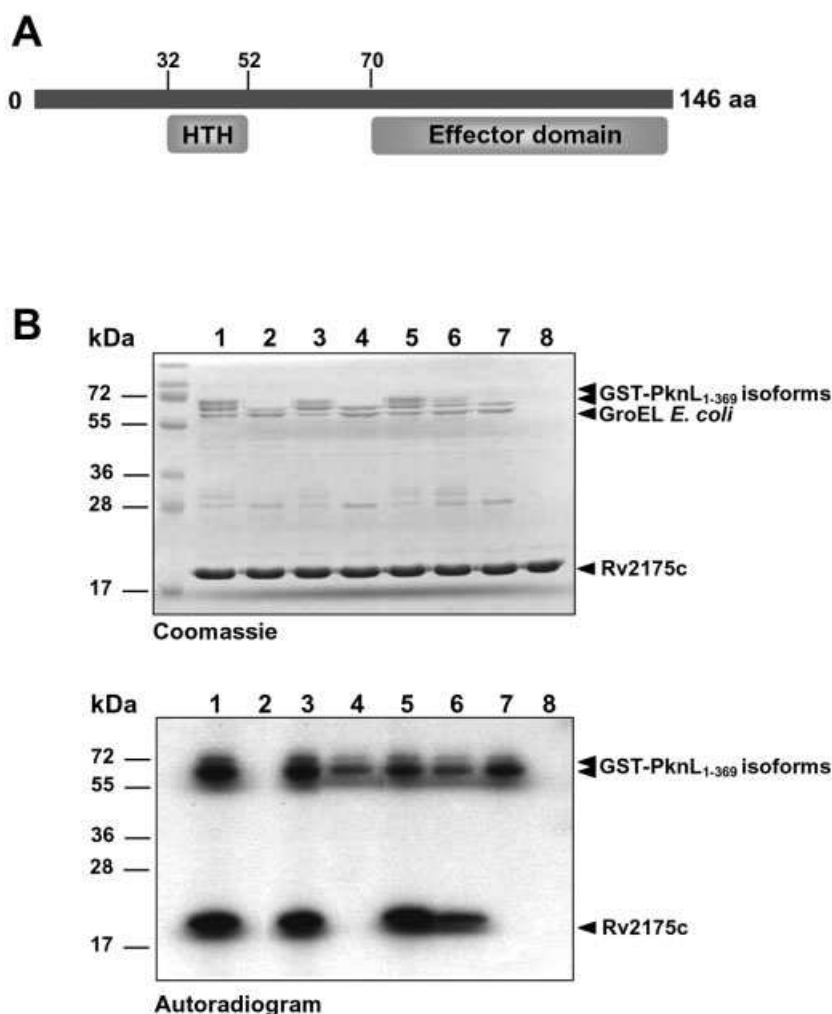


Figure 4. Structural organization of Rv2175c and phosphorylation of Rv2175c by PknL₁₋₃₆₉. (A) Schematic representation of Rv2175c from *M. tb*. Rv2175c encodes a putative DNA-binding transcriptional regulator protein with an N-terminal DNA-binding HTH domain, followed by an effector domain classically found downstream of HTH domains in many regulators. (B) *In vitro* phosphorylation of Rv2175c by PknL₁₋₃₆₉ and the different PknL₁₋₃₆₉ mutants. Recombinant Rv2175c was treated with the TEV protease to remove the N-terminal His-tag and used in phosphorylation assays in the presence of the [γ -³²P]ATP: Rv2175c and PknL₁₋₃₆₉ (lane 1), Rv2175c and PknL₁₋₃₆₉-K48M (lane 2), Rv2175c and PknL₁₋₃₆₉-S171A (lane 3), PknL₁₋₃₆₉-T173A (lane 4), Rv2175c and PknL₁₋₃₆₉-S174A (lane 5), Rv2175c and PknL₁₋₃₆₉-T175A (lane 6), Rv2175c and PknL₁₋₃₆₉-T173A/T175A (lane 7) and Rv2175c alone (lane 8). Proteins were separated by SDS-PAGE and stained with CBB (upper panel), and radioactive bands were revealed by autoradiography (lower panel).

reaction, replacement of Ser171, Ser174 or Thr175 by Ala did not alter PknL-dependent phosphorylation of Rv2175c (Fig. 4B, lower panel, lane 3, 5 and 6). Furthermore, Rv2175c could not be phosphorylated by PknL₁₋₃₆₉-T173A/T175A, clearly demonstrating that phosphorylation of Rv2175c is dependent on Thr173. In PknB, both T171A and T173A mutants interact with the substrate GarA, albeit less efficiently than the wild-type PknB, and retained a partial activity on GarA, indicating that both phosphorylated activation loop threonines are required for optimal PknB-GarA interaction and maximal kinase activity [32]. The finding that Rv2175c interacts only with the phosphorylated form of PknL₁₋₃₆₉ and that this interaction is abolished by the Thr173A substitution of PknL₁₋₃₆₉ suggest that this phosphopeptide recognition motif (TST) is involved in protein-protein interaction between the two partners.

3.7 2-DE profile of Rv2175c phosphorylation *in vivo*

It was recently shown that PknH-mediated *in vitro* phosphorylation leads to five EmbR phosphorylation states [58],

whereas PknB phosphorylated GarA at a single phosphate-acceptor residue [32], and phosphorylated PBPA on two threonines residues [31]. To determine the *in vivo* phosphorylation pattern of Rv2175c, 2-DE of Rv2175c purified from *M. smegmatis* was performed, followed by immunoblotting with specific INDIA HisProbe-HRP, a nickel-activated derivative of HRP, able to directly detect the blotted recombinant His-tagged Rv2175c fusion protein. Western blot analysis showed the presence of three isoforms of Rv2175c, mainly corresponding to the unphosphorylated and mono-phosphorylated forms and, to a lesser extent, to the diphosphorylated species (Fig. 5). The conclusion that the different phosphorylation states of Rv2175c corresponded to mono-, or diphosphorylated forms relies on the fact that each spot on the 2-D gel is equidistant to the next one and can only correspond to a PTM such as phosphorylation. Each phosphate group changes the charge of the protein and makes it to migrate further toward the acidic end of the 2-D strip [25]. Overall, these data strongly suggest that Rv2175c can be phosphorylated *in vivo* on at least one residue.

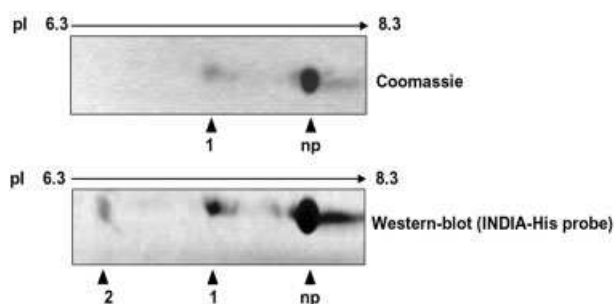


Figure 5. 2-DE profile of *in vivo* phosphorylated Rv2175c. Rv2175c purified from recombinant *M. smegmatis* carrying pVV16-Rv2175c was subjected to 2-DE and stained with CBB (upper panel). Rv2175c isoforms were also detected by immunoblotting using INDIA HisProbe-HRP which recognizes the C-terminal His-tag of the Rv2175c fusion protein (lower panel). The arrowheads represent the different protein isoforms: np, unphosphorylated; 1, monophosphorylated; 2, diphosphorylated.

4 Concluding remarks

The molecular model of PknL/Rv2175c association proposed here relies on direct interactions between the kinase and its substrate. In some cases, the interaction with the docking site on the kinase partner can be mediated by the FHA domain present on the substrate partner [17, 23, 28, 32]. Rv2175c, however, does not possess an FHA domain, and so for this kinase/substrate pair it remains to be addressed which region of Rv2175c interacts with the PknL activation loop *via* Thr173. Structural determination of Rv2175c aimed to decipher the domain(s) interacting with PknL is currently in progress. Further work is also required to investigate whether phosphorylation of Rv2175c is related to its function as a DNA-binding regulator of the *dcw* gene cluster. The conservation in the *Mycobacterium* genus of the *dcw* gene cluster in the vicinity of *pknL/Rv2175c* is also of particular interest and suggests a probable role for this kinase/substrate pair in the regulation of cell division and envelope biosynthesis in mycobacteria. In this context, it is noteworthy that overexpression of PknL₁₋₃₆₉ in recombinant *E. coli* led to the formation of elongated bacteria (data not shown), a morphological phenotype often associated with cell division alteration. This observation substantiates the notion that both *pknL* and *pknB*, which belongs to the same phylogenetical clade, are two kinases that may regulate common physiological processes involved in cell division and cell wall biosynthesis.

This work was supported by grants from the Region Rhone-Alpes (M. C.), the CNRS, the University of Lyon (France) and the National Research Agency (ANR-06-MIME-027-01 to V. M. and L. K.). R. V.-C. is a postdoctoral research fellow from the

Heiser Program for Research in Leprosy and Tuberculosis. The authors wish to thank Professor M. J. Buttner (John Innes Centre, UK) for his helpful comments on the manuscript.

The authors have declared no conflict of interest.

5 References

- [1] Russell, D. G., *Mycobacterium tuberculosis*: Here today, and here tomorrow. *Nat. Rev. Mol. Cell. Biol.* 2001, 2, 569–577.
- [2] Pawson, T., Scott, J. D., Protein phosphorylation in signaling—50 years and counting. *Trends Biochem. Sci.* 2005, 30, 286–290.
- [3] Munoz-Dorado, J., Inouye, S., Inouye, M., A gene encoding a protein serine/threonine kinase is required for normal development of *M. xanthus*, a gram-negative bacterium. *Cell* 1991, 67, 995–1006.
- [4] Zhang, W., Munoz-Dorado, J., Inouye, M., Inouye, S., Identification of a putative eukaryotic-like protein kinase family in the developmental bacterium *Myxococcus xanthus*. *J. Bacteriol.* 1992, 174, 5450–5453.
- [5] Shi, L., Potts, M., Knelly, P. J., The serine, threonine, and/or tyrosine-specific protein kinases and protein phosphatases of prokaryotic organisms: A family portrait. *FEMS Microbiol. Rev.* 1998, 22, 229–253.
- [6] Grangeasse, C., Cozzone, A. J., Deutscher, J., Mijakovic, I., Tyrosine phosphorylation: An emerging regulatory device of bacterial physiology. *Trends Biochem. Sci.* 2007, 32, 86–94.
- [7] Stock, A. M., Robinson, V. L., Goudreau, P. N., Two-component signal transduction. *Annu. Rev. Biochem.* 2000, 69, 183–215.
- [8] Cole, S. T., Brosch, R., Parkhill, J., Garnier, T. *et al.*, Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 1998, 393, 537–544.
- [9] Av-Gay, Y., Everett, M., The eukaryotic-like Ser/Thr protein kinases of *Mycobacterium tuberculosis*. *Trends Microbiol.* 2000, 8, 238–244.
- [10] Peirs, P., De Wit, L., Braibant, M., Huygen, K., Content, J., A serine/threonine protein kinase from *Mycobacterium tuberculosis*. *Eur. J. Biochem.* 1997, 244, 604–612.
- [11] Walburger, A., Koul, A., Ferrari, G., Nguyen, L. *et al.*, Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. *Science* 2004, 304, 1800–1804.
- [12] Deol, P., Vohra, R., Saini, A. K., Singh, A. *et al.*, Role of *Mycobacterium tuberculosis* Ser/Thr kinase PknF: Implications in glucose transport and cell division. *J. Bacteriol.* 2005, 187, 3415–3420.
- [13] Av-Gay, Y., Jamil, S., Drews, S. J., Expression and characterization of the *Mycobacterium tuberculosis* serine/threonine protein kinase PknB. *Infect. Immun.* 1999, 67, 5676–5682.
- [14] Koul, A., Choidas, A., Tyagi, A. K., Drlica, K. *et al.*, Serine/threonine protein kinases PknF and PknG of *Mycobacterium tuberculosis*: Characterization and localization. *Microbiology* 2001, 147, 2307–2314.
- [15] Chaba, R., Raje, M., Chakraborti, P. K., Evidence that a eukaryotic-type serine/threonine protein kinase from *Mycobacterium tuberculosis* regulates morphological changes

- associated with cell division. *Eur. J. Biochem.* 2002, 269, 1078–1085.
- [16] Molle, V., Girard-Blanc, C., Kremer, L., Doublet, P. *et al.*, Protein PknE, a novel transmembrane eukaryotic-like serine/threonine kinase from *Mycobacterium tuberculosis*. *Biochem. Biophys. Res. Commun.* 2003, 308, 820–825.
- [17] Molle, V., Kremer, L., Girard-Blanc, C., Besra, G. S. *et al.*, An FHA phosphoprotein recognition domain mediates protein EmbR phosphorylation by PknH, a Ser/Thr protein kinase from *Mycobacterium tuberculosis*. *Biochemistry* 2003, 42, 15300–15309.
- [18] Nguyen, L., Walburger, A., Houben, E., Koul, A. *et al.*, Role of protein kinase G in growth and glutamine metabolism of *Mycobacterium bovis* BCG. *J. Bacteriol.* 2005, 187, 5852–5856.
- [19] Gopalaswamy, R., Narayanan, P. R., Narayanan, S., Cloning, overexpression, and characterization of a serine/threonine protein kinase pknI from *Mycobacterium tuberculosis* H37Rv. *Protein Expr. Purif.* 2004, 36, 82–89.
- [20] Singh, A., Singh, Y., Pine, R., Shi, L. *et al.*, Protein kinase I of *Mycobacterium tuberculosis*: Cellular localization and expression during infection of macrophage-like cells. *Tuberculosis (Edinb.)* 2006, 86, 28–33.
- [21] Papavinasasundaram, K. G., Chan, B., Chung, J. H., Colston, M. J. *et al.*, Deletion of the *Mycobacterium tuberculosis* *pknH* gene confers a higher bacillary load during the chronic phase of infection in BALB/c mice. *J. Bacteriol.* 2005, 187, 5751–5760.
- [22] Kang, C. M., Abbott, D. W., Park, S. T., Dascher, C. C. *et al.*, The *Mycobacterium tuberculosis* serine/threonine kinases PknA and PknB: Substrate identification and regulation of cell shape. *Genes Dev.* 2005, 19, 1692–1704.
- [23] Grundner, C., Gay, L. M., Alber, T., *Mycobacterium tuberculosis* serine/threonine kinases PknB, PknD, PknE, and PknF phosphorylate multiple FHA domains. *Protein Sci.* 2005, 14, 1918–1921.
- [24] Narayan, A., Sachdeva, P., Sharma, K., Saini, A. K. *et al.*, Serine threonine protein kinases of mycobacterial genus: Phylogeny to function. *Physiol. Genomics* 2007, 29, 66–75.
- [25] Molle, V., Brown, A. K., Besra, G. S., Cozzone, A. J., Kremer, L., The condensing activities of the *Mycobacterium tuberculosis* type II fatty acid synthase are differentially regulated by phosphorylation. *J. Biol. Chem.* 2006, 281, 30094–30103.
- [26] Curry, J. M., Whalan, R., Hunt, D. M., Gohil, K. *et al.*, An ABC transporter containing a forkhead-associated domain interacts with a serine-threonine protein kinase and is required for growth of *Mycobacterium tuberculosis* in mice. *Infect. Immun.* 2005, 73, 4471–4477.
- [27] Thakur, M., Chakraborti, P. K., GTPase activity of mycobacterial FtsZ is impaired due to its transphosphorylation by the eukaryotic-type Ser/Thr kinase, PknA. *J. Biol. Chem.* 2006, 281, 40107–40113.
- [28] Molle, V., Soulat, D., Jault, J. M., Grangeasse, C. *et al.*, Two FHA domains on an ABC transporter, Rv1747, mediate its phosphorylation by PknF, a Ser/Thr protein kinase from *Mycobacterium tuberculosis*. *FEMS Microbiol. Lett.* 2004, 234, 215–223.
- [29] Sharma, K., Gupta, M., Krupa, A., Srinivasan, N., Singh, Y., EmbR, a regulatory protein with ATPase activity, is a substrate of multiple serine/threonine kinases and phosphatase in *Mycobacterium tuberculosis*. *FEBS J.* 2006, 273, 2711–2721.
- [30] Perez, J., Garcia, R., Bach, H., de Waard, J. H. *et al.*, *Mycobacterium tuberculosis* transporter MmpL7 is a potential substrate for kinase PknD. *Biochem. Biophys. Res. Commun.* 2006, 348, 6–12.
- [31] Dasgupta, A., Datta, P., Kundu, M., Basu, J., The serine/threonine kinase PknB of *Mycobacterium tuberculosis* phosphorylates PBPA, a penicillin-binding protein required for cell division. *Microbiology* 2006, 152, 493–504.
- [32] Villarino, A., Duran, R., Wehenkel, A., Fernandez, P. *et al.*, Proteomic identification of *M. tuberculosis* protein kinase substrates: PknB recruits GarA, a FHA domain-containing protein, through activation loop-mediated interactions. *J. Mol. Biol.* 2005, 350, 953–963.
- [33] Greenstein, A. E., Grundner, C., Echols, N., Gay, L. M. *et al.*, Structure/function studies of Ser/Thr and Tyr protein phosphorylation in *Mycobacterium tuberculosis*. *J. Mol. Microbiol. Biotechnol.* 2005, 9, 167–181.
- [34] Greenstein, A. E., Macgurn, J. A., Baer, C. E., Falick, A. M. *et al.*, *M. tuberculosis* Ser/Thr protein kinase D phosphorylates an anti-anti-sigma factor homolog. *PLoS Pathog.* 2007, 3, e49.
- [35] Cole, S. T., Eiglmeier, K., Parkhill, J., James, K. D. *et al.*, Massive gene decay in the leprosy bacillus. *Nature* 2001, 409, 1007–1011.
- [36] Hanks, S. K., Quinn, A. M., Hunter, T., The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science* 1988, 241, 42–52.
- [37] Cohen-Gonsaud, M., Barthe, P., Pommier, F., Harris, R. *et al.*, (1)H, (15)N, and (13)C chemical shift assignments of the resuscitation promoting factor domain of Rv1009 from *Mycobacterium tuberculosis*. *J. Biomol. NMR* 2004, 30, 373–374.
- [38] Jackson, M., Crick, D. C., Brennan, P. J., Phosphatidylinositol is an essential phospholipid of mycobacteria. *J. Biol. Chem.* 2000, 275, 30092–30099.
- [39] Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E. *et al.*, New use of BCG for recombinant vaccines. *Nature* 1991, 351, 456–460.
- [40] Shevchenko, A., Wilm, M., Vorm, O., Mann, M., Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* 1996, 68, 850–858.
- [41] Hanks, S. K., Hunter, T., Protein kinases 6. The eukaryotic protein kinase superfamily: Kinase (catalytic) domain structure and classification. *FASEB J.* 1995, 9, 576–596.
- [42] Kyte, J., Doolittle, R. F., A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 1982, 157, 105–132.
- [43] Yeats, C., Finn, R. D., Bateman, A., The PASTA domain: A beta-lactam-binding domain. *Trends Biochem. Sci.* 2002, 27, 438.
- [44] Zhang, W., Inouye, M., Inouye, S., Reciprocal regulation of the differentiation of *Myxococcus xanthus* by Pkn5 and Pkn6, eukaryotic-like Ser/Thr protein kinases. *Mol. Microbiol.* 1996, 20, 435–447.
- [45] Hanlon, W. A., Inouye, M., Inouye, S., Pkn9, a Ser/Thr protein kinase involved in the development of *Myxococcus xanthus*. *Mol. Microbiol.* 1997, 23, 459–471.

- [46] Motley, S. T., Lory, S., Functional characterization of a serine/threonine protein kinase of *Pseudomonas aeruginosa*. *Infect. Immun.* 1999, *67*, 5386–5394.
- [47] Huse, M., Kuriyan, J., The conformational plasticity of protein kinases. *Cell* 2002, *109*, 275–282.
- [48] Johnson, L. N., Noble, M. E., Owen, D. J., Active and inactive protein kinases: Structural basis for regulation. *Cell* 1996, *85*, 149–158.
- [49] Zheng, X., Papavinasasundaram, K. G., Av-Gay, Y., Novel substrates of *Mycobacterium tuberculosis* PknH Ser/Thr kinase. *Biochem. Biophys. Res. Commun.* 2007, *355*, 162–168.
- [50] Boitel, B., Ortiz-Lombardia, M., Duran, R., Pompeo, F. *et al.*, PknB kinase activity is regulated by phosphorylation in two Thr residues and dephosphorylation by PstP, the cognate phospho-Ser/Thr phosphatase, in *Mycobacterium tuberculosis*. *Mol. Microbiol.* 2003, *49*, 1493–1508.
- [51] Ortiz-Lombardia, M., Pompeo, F., Boitel, B., Alzari, P. M., Crystal structure of the catalytic domain of the PknB serine/threonine kinase from *Mycobacterium tuberculosis*. *J. Biol. Chem.* 2003, *278*, 13094–13100.
- [52] Young, T. A., Delagoutte, B., Endrizzi, J. A., Falick, A. M., Alber, T., Structure of *Mycobacterium tuberculosis* PknB supports a universal activation mechanism for Ser/Thr protein kinases. *Nat. Struct. Biol.* 2003, *10*, 168–174.
- [53] Mann, M., Ong, S. E., Gronborg, M., Steen, H. *et al.*, Analysis of protein phosphorylation using mass spectrometry: Deciphering the phosphoproteome. *Trends Biotechnol.* 2002, *20*, 261–268.
- [54] McLachlin, D. T., Chait, B. T., Analysis of phosphorylated proteins and peptides by mass spectrometry. *Curr. Opin. Chem. Biol.* 2001, *5*, 591–602.
- [55] Annan, R. S., Carr, S. A., Phosphopeptide analysis by matrix-assisted laser desorption time-of-flight mass spectrometry. *Anal. Chem.* 1996, *68*, 3413–3421.
- [56] Loyet, K. M., Stults, J. T., Arnott, D., Mass spectrometric contributions to the practice of phosphorylation site mapping through 2003: A literature review. *Mol. Cell. Proteomics* 2005, *4*, 235–245.
- [57] Molle, V., Zanella-Cleon, I., Robin, J. P., Mallejac, S. *et al.*, Characterization of the phosphorylation sites of *Mycobacterium tuberculosis* serine/threonine protein kinases, PknA, PknD, PknE, and PknH by mass spectrometry. *Proteomics* 2006, *6*, 3754–3766.
- [58] Alderwick, L. J., Molle, V., Kremer, L., Cozzone, A. J. *et al.*, Molecular structure of EmbR, a response element of Ser/Thr kinase signaling in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. USA* 2006, *103*, 2558–2563.

Article II

The *Mycobacterium tuberculosis* Ser/Thr kinase substrate Rv2175c is a DNA-binding protein regulated by phosphorylation

Cohen-Gonsaud M, Barthe P, **Canova MJ**, Stagier-Simon C, Kremer L, Roumestand C, and Molle V.

Journal of Biological Chemistry 2009, In press

Présentation de l'article II :

Depuis leur mise en évidence, différentes études physiologiques ont montré l'implication des STPKs de *M. tuberculosis* dans différentes voies métaboliques comme la synthèse de la paroi, la croissance ou la pathogénie. En revanche, le nombre d'études biochimique portant sur ces STPKs est encore limité et peu de substrats potentiels de ces kinases ont été caractérisés. Suite à l'identification et à la caractérisation du couple kinase/substrat, PknL/Rv2175c, nous nous sommes intéressés à Rv2175c, une protéine conservée chez les actinomycètes mais de fonction encore inconnue. Le but était donc d'identifier la fonction de cette protéine, notamment à partir de l'étude de sa structure par Résonance Magnétique Nucléaire (RMN) et, par la suite, de caractériser le mécanisme de régulation opéré par la phosphorylation sur l'activité de cette protéine. Nous avons ainsi pu déterminer la structure tridimensionnelle de Rv2175c. Cette protéine se présente sous la forme d'un monomère constitué d'une portion flexible (Met1-Ile16) suivie de deux domaines structurés distincts. Le domaine structuré de la portion N-terminale (Pro18-Val77) est composé de deux hélices α et deux feuillets β adoptant un repliement formant un motif original d'Hélice-Tour-Hélice (HTH), motif caractéristique de fixation à l'ADN. Le domaine structuré de la portion C-terminale (Val78-Tyr146), quant à lui, est composé de quatre hélices α anti-parallèles et d'une boucle flexible de 20 résidus d'acides aminés (Thr110-Asn122). Par conséquent, ces données structurales caractérisaient Rv2175c comme une protéine impliquée dans la fixation à l'ADN. Suite à ces observations, des expériences de gel retard et d'anisotropie de fluorescence nous ont permis de confirmer que Rv2175c était effectivement capable de fixer l'ADN.

Nous avons ensuite identifié, en alliant spectrométrie de masse et mutagenèse dirigée, un site unique de phosphorylation correspondant au résidu Thr9 situé au niveau de la portion N-terminale flexible (Met1-Ile16) de la protéine. De manière intéressante, nous avons mis en évidence qu'en remplaçant par mutagenèse dirigée le résidu Thr9 par un résidu « mimant » l'état de phosphorylation (Asp ou Glu), ce mutant Rv2175c T9D ou T9E était incapable de fixer l'ADN. La phosphorylation de Rv2175c sur son résidu Thr9 semble donc réguler son activité de fixation à l'ADN. Il est important de mentionner que cette portion flexible, portant le site unique de phosphorylation, est manquante chez la plupart des espèces de *Mycobacterium* sauf chez *M. tuberculosis*, *M. bovis* et *M. avium paratuberculosis*, suggérant un mécanisme de régulation par phosphorylation original et restreint au complexe *M. tuberculosis*.

The *Mycobacterium tuberculosis* Ser/Thr kinase substrate Rv2175c is a DNA-binding protein regulated by phosphorylation

Martin Cohen-Gonsaud^{1,2,3,*}, Philippe Barthe^{1,2,3}, Marc J. Canova⁴, Charlotte Stagier-Simon^{1,2,3}, Laurent Kremer^{5,6}, Christian Roumestand^{1,2,3} and Virginie Molle^{4,*}

¹CNRS UMR 5048, Centre de Biochimie Structurale, Montpellier, France; ²INSERM U554, Montpellier, France; ³Université Montpellier I et II, Montpellier, France; ⁴Institut de Biologie et Chimie des Protéines, CNRS UMR 5086, Université Lyon 1, IFR128 BioSciences, Lyon-Gerland, Lyon, France; ⁵Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, Universités de Montpellier II et I, CNRS UMR 5235, case 107, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France; ⁶INSERM, DIMNP, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France.

***Corresponding author:**

VM, Tel: (+33) 4 72 72 26 79, Fax: (+33) 4 72 72 26 41, E-mail: vmolle@ibcp.fr

MCG, Tel: (+33) 4 67 41 79 06, Fax: (+33) 4 67 41 79 13, E-mail: martin@cbs.cnrs.fr

Running title: Solution structure of *Mycobacterium tuberculosis* Rv2175c

Keywords: *Mycobacterium*/DNA-binding protein/Rv2175c/solution structure/phosphorylation

Recent efforts have underlined the role of Serine/Threonine Protein Kinases (STPKs) in growth, pathogenesis and cell wall metabolism in *Mycobacterium tuberculosis*. Although most kinases have been investigated for their physiological roles, little information is available regarding how STPK-dependent phosphorylation regulates the activity of kinase substrates. Herein, we focussed on *M. tuberculosis* Rv2175c, a protein of unknown function, conserved in actinomycetes, and recently identified as a substrate of the PknL kinase. We solved the solution structure of Rv2175c by multidimensional NMR and demonstrated that it possesses an original winged Helix-Turn-Helix motif, indicative of a DNA-binding protein. The DNA-binding activity of Rv2175c was subsequently confirmed by fluorescence anisotropy, as well as in electrophoretic mobility shift assays. Mass spectrometry analyses using a combination of MALDI-TOF and LC-ESI/MS/MS identified Thr9 as the unique phosphoacceptor. This was further supported by complete loss of PknL-dependent phosphorylation of a Rv2175c_T9A mutant. Importantly, the DNA-binding activity was

completely abrogated in a Rv2175c_T9D mutant, designed to mimic constitutive phosphorylation, but not in a mutant lacking the first 13 residues. This implies that the function of the N-terminal extension is to provide a phosphoacceptor (Thr9), which following phosphorylation, negatively regulates the Rv2175c DNA-binding activity. Interestingly, the N-terminal disordered extension, which is bearing the phosphoacceptor was found to be restricted to members of the *M. tuberculosis* complex, thus suggesting the existence of an original mechanism which appears to be unique to the *M. tuberculosis* complex.

In response to its environment, *M. tuberculosis* (*M. tb*)¹ activates or represses the expression of a number of genes in order to promptly adjust to new conditions. More precisely, during the infection process, cross-talk of signals between the host and the bacterium take place, resulting in reprogramming the host signaling network. Many of these stimuli are transduced in the bacteria *via* sensor kinases, enabling the pathogen to adapt its cellular response in order to

survive in hostile environments. Although the two-component systems represent the classical prokaryotic mechanism for detection and response to environmental changes, the serine/threonine and tyrosine protein kinases associated with their phosphatases have merged as important regulatory systems in prokaryotic cells (1-3). *M. tb* contains eleven STPKs (4,5) and most are being investigated for their physiological roles and potential application for future drug development to combat tuberculosis (6). Through phosphorylation these STPKs are also thought to play important functions in cell signaling responses as well as in essential metabolic pathways. The cell wall of *M. tb* plays a critical role in the defense of this pathogen in the host and changes in cell wall composition in response to various environmental stimuli are critical to *M. tb* adaptation during infection. Although little is known regarding the cell wall regulatory mechanisms in *M. tb*, there is now an increasing body of evidence indicating that these processes largely rely on STPK-dependent mechanisms (7-9).

Moreover, little information on the range of functions regulated by the STPKs is available and the complicated mycobacterial phosphoproteome is still far from being deciphered. Understanding mycobacterial kinase biology has been severely impeded by the difficulty to identify direct kinase substrates and the subsequent characterization of the phosphorylation site(s). However, several recent studies have reported the identification and characterization of the phosphorylation sites in substrates related to various metabolic pathways in mycobacteria. These include the FHA-containing protein GarA, a key regulator of the tricarboxylic cycle (10,11); PbpA, a penicillin-binding protein required for cell division (12); Wag31, a homologue of the cell division protein DivIVA that regulates growth, morphology and polar cell wall biosynthesis in mycobacteria (13); the β -ketoacyl ACP synthase mtFabH that participates in mycolic acid biosynthesis (9); the anti-anti-sigma factor Rv0516c (14); the alternate sigma factor SigH which is a central regulator of the response to oxidative stress (15); as well as the essential mycobacterial chaperone GroEL1 (16).

Therefore, a further characterization of STPKs substrates is critical in order to unravel mechanisms by which STPK-dependent

phosphorylation induces modifications, thus regulating their activity, ultimately conditioning biological responses in mycobacteria. Such studies may also provide the key to design new inhibitors targeting signal transduction pathways that are specific to *M. tb*.

We recently characterized a novel substrate/kinase pair in *M. tb*, PknL/Rv2175c (17). *pknL* is associated with the ~30 kb *dcw* (*division cell wall*) gene cluster which encompasses several genes involved in cell wall synthesis and cell division (17,18), raising the possibility that PknL might participate in the regulation of this gene cluster. Moreover, *pknL* (Rv2176) is adjacent to the Rv2175c gene, encoding a 16-kDa protein of unknown function. We further demonstrated that phosphorylation of the activation loop Thr173 residue was required for optimal PknL-mediated phosphorylation of Rv2175c. Moreover, Rv2175c belongs to a mycobacterial "core" of 219 genes, identified by macro-array and bioinformatic analysis, common to *M. tb* and *M. leprae* encoding proteins showing no similarity with proteins from other organisms. The presence of Rv2175c as a member of this set of genes emphasises the importance of Rv2175c in the physiology of *M. tb*. In this context, we reasoned that the structural determination of Rv2175c would provide a valuable basis for a better understanding of the function of this protein.

Therefore, we have undertaken the structural determination of Rv2175 using multidimensional nuclear magnetic resonance (NMR) techniques. Herein, we provide strong evidence that Rv2175c is a DNA-binding protein and investigated how phosphorylation of a unique Thr residue in the N-terminal domain of the protein affects its DNA-binding activity.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions -

Strains used for cloning and expression of recombinant proteins were *Escherichia coli* DH5 α (Invitrogen) and *E. coli* BL21(DE3)Star (Novagen). Strains were grown at 37°C in LB medium supplemented with 100 μ g/ml ampicillin

Cloning, expression and purification of Rv2175c and mutant proteins - The Rv2175c gene was amplified by PCR using *M.*

tuberculosis H37Rv genomic DNA as a template and the following primers: #403, 5'- TAT ATA TCG TT CAT ATG CCT GGC CGC GCA CCA GGC TCT -3' (containing a NdeI restriction site underlined) and, #404, 5'- TAT GGA TCC TCA ATA CGC CAT AGC CTG GGC CCG-3' (containing a BamHI restriction site underlined). This 441-bp amplified product was digested by NdeI and BamHI, and ligated into pET15bTev, the variant of pET15b (Novagen) that includes the replacement of the thrombin site coding sequence with a tobacco etch virus (TEV) protease site (19), and in pETPhos, the variant of pET15bTev that is devoid of putative phosphoacceptors in the His-tag fusion (20), thus generating pET15bTev_Rv2175c and pETPhos_Rv2175c respectively. Site-directed mutagenesis was directly performed on pETPhos_Rv2175c using inverse-PCR amplification with the following self-complementary primers: 5'-GGC CGC GCA CCA GGC TCT **GCA** CTT GCG CGG GTG GGC AGC-3' and 5'-GCT GCC CAC CCG CGC AAG TGC AGA GCC TGG TGC GCG GCC-3' for Rv2175_T9A, 5'-GGC CGC GCA CCA GGC TCT **GAC** CTT GCG CGG GTG GGC AGC -3' and 5'- GCT GCC CAC CCG CGC AAG **GTC** AGA GCC TGG TGC GCG GCC -3' for Rv2175_T9D, 5'- GGC CGC GCA CCA GGC TCT **GAA** CTT GCG CGG GTG GGC AGC-3' and 5'-GCT GCC CAC CCG CGC AAG **TTC** AGA GCC TGG TGC GCG GCC-3' for Rv2175_T9E (the corresponding substitutions are shown in bold). The Rv2175c N-terminal truncated fragment lacking the first 13 residues was amplified by PCR using *M. tuberculosis* H37Rv genomic DNA as a template and the following primers: #615, 5'-TAA TAG CTC ATA TGG GCA GCA TTC CCG CTG GCG ATG AC-3' (containing a NdeI restriction site underlined) and, #404. This 402-bp amplified product was digested by NdeI and BamHI, and ligated into pET15bTev thus generating pET15bTev_Rv2175c₁₄₋₁₄₆. All constructs were verified by DNA sequencing. Recombinant *E. coli* BL21(DE3)Star strains harbouring the Rv2175c-expressing constructs pET15bTev_Rv2175c, pETPhos_Rv2175c, pETPhos_Rv2175c_T9A, pETPhos_Rv2175c_T9D, pETPhos_Rv2175c_T9E and pET15bTev_Rv2175c₁₄₋₁₄₆ were used to inoculate 750 ml of LB medium supplemented

with ampicillin and incubated at 37°C with shaking. When A_{600} reached 0.6, IPTG was added at a final concentration of 0.2 mM, and growth was continued for an additional 3 h at 37°C with shaking. Purifications of the His-tagged proteins were performed as reported previously (8,21). When required, the protein was treated with TEV protease. Finally, the purified protein was concentrated and applied to a Superdex 75 26/60 (Amersham biosciences) size exclusion column, equilibrated in buffer 20 mM Na-Acetate pH 4.6, 300 mM NaCl in order to remove any remaining impurities and the cleaved tag when TEV protease cleavage was performed. The purified Rv2175c protein were identified by SDS-PAGE and stored at -20°C until required. This protocol was carried out for all the non-labelled constructs and ¹⁵N labelled of wild type and mutant Rv2175c constructs, excepted that the cultures were grown in a minimum media containing ¹⁵NH₄Cl and ¹³C₆ glucose as the sole nitrogen and carbon sources.

Cloning, expression and purification of the recombinant PknL₁₋₂₈₁ protein - The *pknL* PCR fragment encoding the kinase core domain (CD) corresponding to the kinase domain without the juxtamembrane linker of PknL (residues 1–281 out of 399) was amplified by PCR using *M. tuberculosis* H37Rv genomic DNA as a template and the following primers: #399, 5'-TAT CAT ATG CCG TTG GAG AGC GCG CTG CTG -3' (containing a NdeI restriction site underlined) and, #400, 5'-TAT GGA TCC TTA CTC CTC GGC GAT CGC CTC CAG -3' (containing a BamHI restriction site underlined). This 843-bp amplified product was digested by NdeI and BamHI, and ligated into pET15bTev. *E. coli* BL21(DE3)Star cells were transformed with the pET15bTev_ *pknL*₁₋₂₈₁ vector expressing the PknL₁₋₂₈₁ core domain. Purifications of the His-tagged protein was performed as above (8,21). When required, the protein was treated with TEV protease according to the manufacturer's instructions (Invitrogen).

Solution structure of Rv2175c - NMR experiments were carried out at 14.1 Tesla on a Bruker Avance 600 spectrometer equipped with 5 mm z-gradient ¹H-¹³C-¹⁵N triple resonance cryoprobe. ¹H, ¹³C and ¹⁵N resonances were assigned using standard triple-resonance three-dimensional experiments (22) recorded at 30°C on

0.3 mM ^{15}N - or $^{15}\text{N},^{13}\text{C}$ -labeled Rv2175c protein samples dissolved in 10 mM Na-Acetate, pH 4.6, 150 mM NaCl with 5% D_2O for the lock. ^1H chemical shifts were directly referenced to the methyl resonance of DSS, while ^{13}C and ^{15}N chemical shifts were referenced indirectly to the absolute frequency ratios $^{15}\text{N}/^1\text{H} = 0.101329118$ and $^{13}\text{C}/^1\text{H} = 0.251449530$. All NMR spectra were processed with GIFA (23). NOE peaks identified on 3D [$^1\text{H},^{15}\text{N}$] and [$^1\text{H},^{13}\text{C}$] 3D NOESY-HSQC were assigned through automated NMR structure calculations with CYANA 2.1 (24). Backbone ϕ and ψ torsion angle constraints were obtained from a database search procedure on the basis of backbone (^{15}N , HN, $^{13}\text{C}'$, $^{13}\text{C}^\alpha$, H^α , $^{13}\text{C}^\beta$) chemical shifts using the program TALOS (25). Hydrogen bond restraints were derived using standard criteria on the basis of the amide $^1\text{H}/^2\text{H}$ exchange experiments and NOE data. When identified, the hydrogen bond was enforced using the following restraints: ranges of 1.8-2.3 Å for $d(\text{N-H},\text{O})$, and 2.7-3.3 Å for $d(\text{N},\text{O})$. The final list of restraints (from which values redundant with the covalent geometry has been eliminated) consisted of 477 intra-residues, 576 sequential, 278 medium-range ($1 < i-j \leq 4$), and 345 long-range upper bound distance restraints, 190 backbone dihedral angle restraints (ϕ and ψ) and 66 hydrogen bond restraints. The 30 best structures (based on the final target penalty function values) were minimized with CNS 1.2 according the RECOORD procedure (26) and analyzed with PROCHECK (27). The *r.m.s.* deviations were calculated with MOLMOL (28). Structural statistics are given in Table 1.

Electrophoretic mobility shift assays - The DNA fragment used in this assay corresponds to 200 bp of non-relevant double-stranded *M. tuberculosis* DNA. The 5' ends of DNA were labelled using [γ - ^{32}P]-ATP and T4 polynucleotide kinase. A typical assay mixture contained in 20 μl : 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 5% (v/v) glycerol, 0.5 mg of BSA, radioactive DNA probe (2000 cpm. ml^{-1}) and various amounts of the purified Rv2175c proteins. After 30 min of incubation at room temperature, 20 μl of this mixture was loaded onto a native 4% (w/v) polyacrylamide TBE Ready Gel (Bio-Rad) and electrophoresed in 1% TBE buffer for 1 h at 100 V. cm^{-1} . Radioactive species were detected by autoradiography after exposure using direct exposure to films.

Fluorescence anisotropy - The 23-bp oligo nucleotide with a 3' reactive amine group (sequence 5'-CTC CAG GTC ACT GTG ACC TCC TC-3') was purchased from Sigma Genosys. It was covalently labeled with Alexa Fluor 488 succinimidyl ester dye (Invitrogen). A 10-fold molar excess of the dye was added to a solution of DNA in 0.1M Sodium Borate pH9 buffer and the reaction was allowed to proceed at room temperature for 3 hr with continuous agitation. The reaction was stopped by adding 10% Tris-HCl 1M. After ethanol precipitation, the labeled oligonucleotide was further purified using reverse phase chromatography on a $\text{C}_2\text{-C}_{18}$ column. Finally, it was hybridized with a 1.1 / 1 molar excess of complementary strand. Steady-state fluorescence anisotropy binding titrations were carried out on a Tecan Sapphire II microplate reader, using a 470nm LED for excitation, and a monochromator set at 530 nm (bandwidth 20 nm) for emission.

In vitro kinase assays - *In vitro* phosphorylation of PknL and Rv2175c proteins was carried out for 30 min at 37°C in a reaction mixture (20 μl) containing buffer P (25 mM Tris-HCl, pH 7.0; 1 mM DTT; 5 mM MgCl_2 ; 1 mM EDTA) with 200 $\mu\text{Ci}/\text{ml}$ [γ - ^{32}P]-ATP. Phosphorylation of Rv2175c by PknL₁₋₂₈₁ was performed with 3 μg of Rv2175c in 20 μl of buffer P with 200 $\mu\text{Ci}/\text{ml}$ [γ - ^{32}P]-ATP and 500 ng of PknL₁₋₂₈₁ for 30 min at 37°C. The reaction was stopped by addition of an equal volume of 2X sample buffer and the mixture was heated at 100°C for 5 min. After electrophoresis, gels were soaked in 16% TCA for 10 min at 90°C, and dried. Radioactive proteins were visualized by autoradiography using direct exposure to films.

In vitro phosphorylation for NMR, electrophoretic mobility shift assays, and mass spectrometry analysis was performed as described above except that [γ - ^{32}P]-ATP was replaced by 5 mM non-radioactive ATP and incubated overnight.

Mass spectrometry analysis - Purified Rv2175c was subjected to *in vitro* phosphorylation by PknL₁₋₂₈₁ as described above with 5 mM cold ATP. Subsequent mass spectrometry analyses were performed as previously described (29).

RESULTS

Rv2175c solution structure - Rv2175c, a 146-aminoacid protein with a calculated molecular mass of 16 kDa exhibits very weak similarity to transcriptional regulatory proteins harbouring a DNA-binding domain (residues 32 to 52) with a Helix-Turn-Helix (HTH) motif, as well as homologies to α -helices protein like cyclines and cytochromes (17). Since bioinformatic analyses using several different web-based meta-servers failed to clearly identify Rv2175c as a typical DNA-binding protein, we decided to determine its structure, expecting that it would provide new insights with respect to its function. Rv2175c was isotopically labelled with ^{15}N , and ^{15}N - ^{13}C , expressed and purified from *E. coli* carrying pET15bTev_Rv2175c. Following removal of its N-terminal His-tag using TEV protease, purified Rv2175c was used for the structural determination by multidimensional nuclear magnetic resonance spectroscopy (NMR). By combining the information from the double- and triple-resonance heteronuclear experiments, we were able to assign all the amide group resonances (except Thr29) for the non-proline residues (12 prolines), 93.8 % of the other backbone resonances ($\text{C}\alpha$, C' and $\text{H}\alpha$), 76.7 % of the $\text{C}\beta$ resonances and about 96 % of the side chain protons.

NMR experiments revealed that the N-terminal part (Met1-Ile16) and a large loop (Thr110-Asn122) of Rv2175c were fully disordered. The corresponding residues gave rise to the intense cross-peaks centred at 8.5 ppm in the HSQC spectrum (Fig. 1). Intriguingly, these two regions correspond to the less conserved parts of the protein found in different actinomycetes homologous proteins. Noteworthy, the loop Thr110-Asn122 is present in mycobacteria but missing constantly in *Corynebacterium* or *Streptomyces* despite a sequence identity close to 60% for the core residues (Fig. 2). In addition, multiple sequence alignments of Rv2175c homologues revealed that, except for *M. tuberculosis* and *M. bovis*, most homologues, including *M. smegmatis*, lack the first twelve residues (Fig. 2). One notable exception is *M. avium paratuberculosis*, which carries a 20-aminoacid extension with respect to the *M. smegmatis* sequence.

We solved the structure of Rv2175c, which corresponds to a monomer constituted of two domains with six α -helices and two β -strands in total (Fig. 3A and 3B) with flexibility existing between the N-terminal (residues Pro18 to Val77) and the C-terminal domains (residues Val78 to Tyr146). The thirty best structures were calculated and superimposed for the whole protein heavy atoms (except the first 16 residues) with a root mean square deviation (r.m.s.d.) of 1.1Å while the r.m.s.d. calculated for the N-terminal or the C-terminal domain were of 0.7Å and 0.85Å, respectively (Fig. 4). Our data indicate that the N-terminal domain, with the topological $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ order, harbours an unusual prokaryotic winged Helix-Turn-Helix (wHTH) DNA-binding motif missing the typical third helix (30) (Fig. 3A and 3B). To our knowledge, the only characterized HTH DNA-binding proteins missing the third helix are the bacteriophage protein from the Xis Nucleoprotein Filament (31). In the Rv2175c N-terminal domain, the turn following the second strand allows the next six residues (Ile66 and Phe71) to cap the hydrophobic core formed by the two first helices of the wHTH, a role usually devoted to the third helix of the motif (Fig. 3C). Moreover, the Rv2175c C-terminal domain is composed of four anti-parallel helices ($\alpha 3$ - $\alpha 6$) and their connecting turns together with a mobile 20 amino-acid loop (residues Thr110-Asn122). If most of the hydrophobic residues are buried in the helix bundle, some remain partially solvent exposed (Leu87, Val138 and Tyr146). Interestingly, in transcriptional regulators harbouring a wHTH domain, the C-terminal segment usually possesses a dual role, a functional role as regulatory domain and a structural role as an actor of protein dimerization when bound to DNA. The relative positioning of these two domains is dictated by contacts between the hydrophobic surface encompassing the residues from the C-terminal α -helices $\alpha 4$ (Ile98, Met99 and Phe103) and $\alpha 5$ residues (Val126 and Leu129) and the N-terminal residues Val22 and Phe70.

Overall, the NMR solution structure determination of Rv2175c clearly identified structural features typically found in DNA-binding proteins with an original N-terminal wHTH domain as well as a C-terminal regulatory domain of unknown function.

Rv2175c is a DNA-binding protein - To confirm that Rv2175c is a DNA-binding protein two biochemical strategies were used. First, we confirmed by electrophoretic mobility shift assay (EMSA) the ability of Rv2175c to bind non-relevant double-stranded DNA at low salt concentration. Since the specific Rv2175c DNA target(s) remain unknown, the binding experiments were carried out using in a typical assay mixture containing 50 mM NaCl with increasing amounts of purified Rv2175c. The results indicated that the Rv2175c protein interacted measurably with double-stranded DNA (Fig. 5A). An excess of cold DNA was also mixed along with the labelled probe and the absence of a shifted probe indicated specificity of the binding activity of Rv2175c towards the probe.

DNA-binding activity of Rv2175c was further analyzed by fluorescence anisotropy determination, a method of choice to measure interaction between different molecules like protein/DNA. A 30-bp DNA segment was labelled with alexa Fluor 488 dye and mixed with unlabelled Rv2175c. As expected, in the absence of salt, Rv2175c exhibited a substantial DNA-binding activity (Fig. 5C). However, increasing NaCl concentrations from 0 to 150 mM resulted in dramatic decrease in the affinity for DNA, with a complete loss of activity culminating at 100 mM NaCl (Fig. 5C). The addition of salt raises the DNA-protein specificity and demonstrates that the anisotropy increase observed at low salt with the highest protein concentration is due to proper DNA-protein interactions, corresponding to a regain of specificity, rather than to a non-specific aggregation. Taken together, our structural and biochemical data clearly establish the function of Rv2175c as a DNA-binding protein.

Rv2175c is phosphorylated on a unique threonine residue - We recently showed that Rv2175c was exclusively phosphorylated on Thr residues, *in vitro* as well as *in vivo*, but the phosphorylation sites could not be identified (17). Therefore, in order to decipher the role of the phosphorylation event it appeared necessary to identify which of the 8 threonines of Rv2175c corresponded to the phosphorylated site(s). Thus, recombinant Rv2175c was incubated with cold ATP in the presence of PknL, and subjected to mass spectrometry analysis after tryptic

digestion. ProteinPilot® database searching software (version 2.0, Applied Biosystems), using the Paragon method with phosphorylation emphasis, was used to detect and identify the phosphorylated peptides. The sequence coverage of the protein was 91% and phosphorylation occurred only on peptide [5-12] with an 80 Da mass increment from 771.42 to 851.40 Da (monoisotopic mass). The MS/MS spectrum of the corresponding doubly charged ion at m/z 426.7 unambiguously confirmed the presence of the phosphate group on the threonine residue T9 (Fig. 6A), in agreement with our previous phosphoamino analysis, which indicated that phosphorylation of Rv2175c occurred exclusively on Thr residues (17).

Definitive identification of Thr9 as being the unique phosphorylation site in Rv2175c was achieved by site-directed mutagenesis to introduce a mutation that prevents specific phosphorylation (Thr9 to Ala9 replacement). This mutant was expressed, purified as a His-tagged protein in *E. coli* BL21(DE3)Star harboring pETPhos_Rv2175c_T9A and used in an *in vitro* kinase assay. The recombinant Rv2175c_T9A was incubated along with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and PknL₁₋₂₈₁. The mixture was separated by SDS-PAGE and analyzed by autoradiography. As shown in Fig. 6B (upper panel), equal amounts of Rv2175c or mutant Rv2175c_T9A were used. Phosphorylation of Rv2175c_T9A was completely abrogated, compared to phosphorylation of Rv2175c, as evidenced by the absence of a specific radioactive band (Fig. 6B, lower panel). These results unambiguously demonstrate that Rv2175c_T9A has lost its ability to be phosphorylated by PknL. An additional round of mass spectrometry analysis was also performed directly on Rv2175c_T9A pre-treated with ATP and PknL, which failed to identify any additional phosphate group that could eventually have arisen as a compensatory mechanism to the loss of the Thr9 phosphorylation (data not shown).

Phosphorylation on the Rv2175c N-terminal extension modulates its DNA-binding activity - The structural elucidation of Rv2175c as a DNA-binding protein and the identification of its phosphorylation site led to the hypothesis that phosphorylation may influence the DNA-binding activity of Rv2175c. Following a strategy which has been successfully used to demonstrate that

regulation of a substrate protein *via* phosphorylation is important during active cell growth in mycobacteria (Wag31), or for mycolic acid metabolism (mtFabH) (9,13), we expressed and purified the phosphorylation mimics Rv2175c_T9D or Rv2175c_T9E. Indeed, previous studies have shown that acidic residues such as Asp or Glu qualitatively recapitulate the effect of phosphorylation with regard to functional activity (32). The option was taken to analyse and compare the DNA-binding activity of Rv2175c, Rv2175c_T9D or Rv2175c_T9E using non-relevant DNA by EMSA and by fluorescence anisotropy at various NaCl concentrations.

As mentioned above, the EMSAs were carried out using a radioactive 200 bp non-relevant DNA fragment, except that Rv2175 was substituted by Rv2175c_T9D or Rv2175c_T9E (data not shown). While Rv2175c protein interacted with this double-stranded DNA, the Rv2175c_T9D and Rv2175c_T9E phosphorylation mimics failed to shift the probe, indicating that they had lost their ability to bind to DNA (Fig. 5A). On the contrary, EMSA carried out using a Rv2175c_T9A mutant, a mutation that prevents the phosphorylation of Rv2175c, showed that this mutant retained its DNA-binding activity (data not shown), thus confirming the critical role of phosphorylation in Rv2175c to inhibit the DNA binding. Moreover, EMSA assays were performed using Rv2175c phosphorylated by PknL in presence of cold ATP, thus generating pT9-Rv2175c. As shown in Fig. 5B, the phosphorylated isoform of Rv2175c failed to bind DNA, thus confirming the critical role of phosphorylation to prevent DNA binding, as previously observed with the mimics mutants, T9D or T9E (Fig. 5A).

In addition, the fluorescence anisotropy method using the labelled 30-bp DNA fragment, which was mixed with unlabelled Rv2175c, Rv2175c_T9D, or Rv2175c_T9E was performed in order to confirm the phosphorylation role in Rv2175c DNA-binding activity. As shown in Fig. 5C, Rv2175c exhibited a strong DNA-binding activity in the absence of salt, as expected while the affinity of Rv2175c_T9D for DNA in the absence of salt was comparable to that of the Rv2175c at 100 mM NaCl (Fig. 5D). Similar results were observed when Rv2175c_T9D was replaced by the other mimic mutant Rv2175c_T9E (data not shown).

Together, these results indicate that mimicking phosphorylation by an acid residue at position 9 abrogates the DNA-binding activity of Rv2175c. This highlights the critical role of Thr9, which following phosphorylation, negatively controls the DNA-binding activity of Rv2175c. This prompted us to investigate whether phosphorylation alters the structure of Rv2175c using 2D and 3D NMR experiments on the mimic mutants. However, our different analyses did not reveal major differences on a structural basis compared to the non-phosphorylated isoform of Rv2175c protein.

To investigate whether the N-terminal extension characterizing the *M. tb* protein was contributing to the DNA-binding activity of Rv2175c, fluorescence anisotropy of the Rv2175c mutant lacking the N-terminal extension (Rv2175c₁₃₋₁₄₆) was recorded using the same conditions as mentioned above. This truncated protein exhibited a DNA-binding activity comparable to the one of full-length protein (data not shown), indicating that the first thirteen N-terminal residues are not involved in the DNA-binding activity of the protein in the absence of phosphorylation, but act as a phosphoacceptor site that regulates Rv2175c DNA-binding activity.

Effect of Thr9 phosphorylation on Rv2175c structural organization - In a second set of experiments, PknL was purified and used to phosphorylate a ¹⁵N labelled sample of Rv2175c. The first series of experiment were performed in the same conditions at pH 4.6 and few chemical shift variations could be observed on the [¹H,¹⁵N] HSQC spectrum recorded after treatment with the kinases (Fig. 7A and 7B). Only the Thr9 residue and its close neighbours could be clearly detected, while no significant modifications of the Rv2175c core domain could be resolved by NMR for the phosphorylated isoform of Rv2175c in these conditions (Fig. 7B). Importantly, frequency resonance sequential attribution revealed a unique phosphorylation site on Thr9, clearly identified as the most important chemical shift change, thus confirming our mass spectrometry analysis (Fig. 6A), and that PknL was clearly able to phosphorylate Rv2175c despite an obvious structural shift that could explain the role of Thr9 phosphorylation. The down-field chemical shift change from Thr to pThr observed at pH

4.6 was around 0.5 ppm, instead of the 2 ppm we observed in recent studies made at a higher pH 6.4 (21). Therefore, in order to determine whether the pH could be responsible for the failure to observe a structural shift, five samples of the protein were prepared at pH 4.6, 5, 5.3, 6.1, 6.4 or 6.8. However, due to instability as the pH increased, around 90% of the protein precipitated. Consequently; only limited NMR data were obtained, particularly the [¹H,¹⁵N] HSQC experiment using different acquisition time. Although we could not record any other experiment to solve and compare the solution structures of the phosphorylated and non-phosphorylated isoforms at different pH, it is noteworthy that only the residue Thr (with a 1.5 ppm chemical shift) and its close neighbours were affected by phosphorylation without modifications of the core domain residues, indicating that pH did not affect any structural rearrangement (Fig. 7C and 7D). It appears therefore tempting to hypothesize that PknL-dependent phosphorylation on Thr9 does not engender major structural modifications on the Rv2175c monomer. However, it remains possible that the inhibitory mechanism triggered by phosphorylation may disturb protein dimerisation and/or induce a structural rearrangement between the N-terminal and the C-terminal domains, or an interaction with a third partner beside DNA.

DISCUSSION

Recent years have been characterized by a tremendous increase in structural information on protein-DNA complexes and uncovered a remarkable structural diversity in DNA binding folds (33). A subclass of the HTH family known as the winged HTH (wHTH) proteins are so named due to the presence of an additional wing immediately C-terminal to the HTH unit that mediates additional contacts with the DNA (30). Here, we have solved the solution structure of Rv2175c, which is characterized by the presence of an original N-terminal wHTH (winged Helix-Turn-Helix). This domain is associated with a putative regulatory C-terminal domain sharing no similarities with any structure solved to date. All structural features necessary for protein-DNA interactions are present, except the third helix of the wHTH tri-helical bundle domain which is missing in Rv2175c, like in the

bacteriophage protein from the Xis Nucleoprotein Filament (31), while the first two helices and the winged β -strand hairpin corresponding to the domains of the protein that directly interacts with the DNA are conserved.

Biochemical studies including EMSA and fluorescence anisotropy further confirmed that Rv2175c is capable to bind DNA at low salt concentrations. Taken, collectively, our data clearly establish the function of Rv2175c as a DNA-binding protein. Our biochemical studies uncovered that Thr9, laying within the N-terminal disordered extension, as the unique phosphoacceptor in Rv2175c. The presence of the phosphorylation site in a flexible region of the protein is likely to promote optimal presentation/interaction between the phosphorylation site and the kinase.

This work presents compelling evidence that *Rv2175c* encodes a DNA-binding protein and that this activity is inherent to the main core domain of the protein as a truncated version of Rv2175c protein lacking the first 13 residues presented the same DNA-binding properties than the wild-type Rv2175c. Importantly, Rv2175c mutants mimicking the phosphorylation isoform failed to express DNA-binding activity, supporting the view that phosphorylation of Thr9 negatively regulates Rv2175c activity. Surprisingly structural comparison of the non-phosphorylated and PknL-phosphorylated isoforms of Rv2175c did not reveal major conformational changes. Our structural study revealed that Rv2175c behaves as a monomer in solution, a feature that may explain the failure to detect major structural changes. Since DNA-binding proteins, and particularly prokaryotic transcription factors, usually act as homodimers, we could speculate that phosphorylation of Thr9 interferes with cooperativity and/or oligomerization mechanisms linked to the DNA-binding of a dimeric form of Rv2175c. Whether phosphorylation exerts its inhibitory effect on DNA binding by disrupting homotypic interactions between two monomers represents an attractive hypothesis. However, as a prerequisite to test this hypothesis, specific DNA target(s) of Rv2175c will have to be identified. Work is currently in progress to identify the putative regulon(s) controlled by Rv2175c using a chromatin immunoprecipitation (Chip-on-chip) approach, which should lead to the Rv2175c

specific DNA targets and to determine their locations on a whole-genome basis.

Multiple sequence alignments indicated that the disordered N-terminal sequence bearing the phosphoacceptor is only restricted to a few pathogenic mycobacterial species. In addition to *M. tb* and *M. bovis* a full conservation of this domain was found to be present in *M. africanum*, *M. microti* and *M. canetti* (data not shown), but not in other pathogenic species such as *M. marinum* or *M. leprae* or in non-pathogenic species such as *M. smegmatis*. Overall, these observations support the occurrence of a regulation mechanism by phosphorylation of Rv2175c, which is unique to the *M. tuberculosis* complex. During the infection process, *M. tb* encounters numerous environmental conditions and induces or represses a number of genes for a quick adjustment to new conditions allowing the bacteria to adapt and survive within its host. Whether the phosphorylation-dependent mechanism controlling the activity of Rv2175c contributes to the adaptation of *M. tb* in its host requires to be further studied.

Because these organisms display a complex life style comprising different environments and developmental stages, it is believed that their success results from their remarkable capacity to survive within the infected host, which is also linked to the presence of an unusual cell wall. It is therefore tempting to speculate that the PknL/Rv2175c couple participates in the modulation and adaptation of cell wall components in response to environmental changes. In this context it is important to mention that this kinase/substrate pair is present in the close vicinity of the *dcw* (division and cell wall synthesis) gene cluster, encompassing several genes involved in cell wall synthesis and cell division (17,18). In addition, Kang *et al.* demonstrated that the activity of Wag31, a homologue of the cell division protein DivIVA, is controlled by phosphorylation by mycobacterial STPKs and that Wag31 regulates cell shape and cell wall synthesis in *M. tb* in response to environmental signals mediated via STPK phosphorylation of Wag31. Depletion of Wag31 causes a dramatic morphological change in which one end of the cell becomes round rather than rod-shaped (34). Interestingly, *wag31* (Rv2145c) belongs to the 30 kb *dcw* cluster. Whether Rv2175c acts as a transcriptional regulator involved in controlling the *dcw* gene

cluster has not been elucidated yet. Access to the DNA target(s) of Rv2175c may help to understand how regulation via phosphorylation of Rv2175c in response to environmental changes contributes to the persistence of *M. tb* within the infected host. A detailed characterization of the regulon(s) will be a major step in defining the role(s) of Rv2175c in the *M. tb* physiological adaptation to its environment.

In prokaryotes, protein kinases can be classified into two super families based on their sequence similarities and enzymatic specifications: (i) the histidine kinase superfamily, which belongs to the two-component systems (1). In this system, the stimulus causes the histidine kinase to autophosphorylate a conserved histidine residue, forming a highly reactive phosphoramidate bond. The cognate response regulator catalyzes the phosphoryl transfer to a conserved aspartate within its own receiver domain. The phosphorylated response regulator interacts with transcription factors, which in turn, up or down regulate number of genes; and (ii) the superfamily of serine, threonine, and tyrosine kinases (2,3). *M. tb* has only twelve paired two-component systems homologues compared with more than 30 present in *E. coli* or *Bacillus subtilis*, which may be a consequence of the presence of a relatively much higher number of STPKs in *M. tb* (4). In addition to the proposed roles of STPKs in pathogenesis and development, it is likely that some STPKs simply fulfil the role of the classical bacterial two-component system regulatory proteins. In this context, the PknL/Rv2175c pair, presents functional analogy with two-component systems, in that PknL can be compared to the sensor kinase, and Rv2175c as the transcriptional factor controlled by phosphorylation. In support of this view, PknH has been shown to phosphorylates EmbR (encoded by its adjacent gene), a putative transcriptional regulator of arabinosyl transferases EmbCAB (35,36). Activation of EmbR upon phosphorylation by PknH induces transcription from the *embCAB* operon, leading to higher lipoarabinomannan/lipomannan ratio (37). Moreover, in a recent report, it was demonstrated that PknK (Rv3080c) phosphorylates the AraC/XylS transcriptional regulator VirS (Rv3082c), which regulates expression of the mycobacterial monooxygenase (*mymA*) operon and that PknK-mediated

phosphorylation of VirS increase its affinity for *mym* promoter DNA (38). These few examples highlight the functional analogy of *M. tb* STPKs in regulating the activity of DNA-binding proteins in a way similar to the classical two-component couples.

In conclusion, our study clearly established the function of Rv2175c as a DNA-binding protein, whose activity is controlled by

phosphorylation of a unique residue in the N-terminal domain of the protein. However, future work is required to identify and characterize the DNA target(s) of Rv2175c and its possible link with the *dcw* gene cluster. In combination with structural analysis of the PknL/Rv2175c complex, these studies may also provide the key to design molecules for selective disruption of signal transduction that are specific to *M. tb*.

Accession Numbers.

The structure coordinates for Rv2175c have been deposited in the Protein Data Bank as entry 2KFS. The chemical shifts have been deposited in the BioMagResBank under the accession number BMRB-16188.

REFERENCES

1. Stock, J. B., Ninfa, A. J., and Stock, A. M. (1989) *Microbiol Rev* **53**, 450-490
2. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) *Science* **241**, 42-52
3. Hunter, T. (1995) *Cell* **80**, 225-236
4. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., 3rd, Tekaiia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) *Nature* **393**, 537-544
5. Av-Gay, Y., and Everett, M. (2000) *Trends Microbiol* **8**, 238-244
6. Wehenkel, A., Bellinzoni, M., Grana, M., Duran, R., Villarino, A., Fernandez, P., Andre-Leroux, G., England, P., Takiff, H., Cervenansky, C., Cole, S. T., and Alzari, P. M. (2008) *Biochim Biophys Acta* **1784**, 193-202
7. Sharma, K., Gupta, M., Krupa, A., Srinivasan, N., and Singh, Y. (2006) *Febs J* **273**, 2711-2721
8. Molle, V., Brown, A. K., Besra, G. S., Cozzzone, A. J., and Kremer, L. (2006) *J Biol Chem* **281**, 30094-30103
9. Veyron-Churlet, R., Molle, V., Taylor, R. C., Brown, A. K., Besra, G. S., Zanella-Cleon, I., Futterer, K., and Kremer, L. (2008) *J Biol Chem*
10. O'Hare, H. M., Duran, R., Cervenansky, C., Bellinzoni, M., Wehenkel, A. M., Pritsch, O., Obal, G., Baumgartner, J., Vialaret, J., Johnsson, K., and Alzari, P. M. (2008) *Mol Microbiol* **70**, 1408-1423
11. Villarino, A., Duran, R., Wehenkel, A., Fernandez, P., England, P., Brodin, P., Cole, S. T., Zimny-Arndt, U., Jungblut, P. R., Cervenansky, C., and Alzari, P. M. (2005) *J Mol Biol* **350**, 953-963
12. Dasgupta, A., Datta, P., Kundu, M., and Basu, J. (2006) *Microbiology* **152**, 493-504
13. Kang, C. M., Nyayapathy, S., Lee, J. Y., Suh, J. W., and Husson, R. N. (2008) *Microbiology* **154**, 725-735
14. Greenstein, A. E., Macgurn, J. A., Baer, C. E., Falick, A. M., Cox, J. S., and Alber, T. (2007) *PLoS Pathog* **3**, e49
15. Park, S. T., Kang, C. M., and Husson, R. N. (2008) *Proc Natl Acad Sci U S A* **105**, 13105-13110
16. Canova, M. J., Kremer, L., and Molle, V. (2009) *J Bacteriol*
17. Canova, M. J., Veyron-Churlet, R., Zanella-Cleon, I., Cohen-Gonsaud, M., Cozzzone, A. J., Becchi, M., Kremer, L., and Molle, V. (2008) *Proteomics* **8**, 521-533

18. Narayan, A., Sachdeva, P., Sharma, K., Saini, A. K., Tyagi, A. K., and Singh, Y. (2007) *Physiol Genomics* **29**, 66-75
19. Cohen-Gonsaud, M., Barthe, P., Pommier, F., Harris, R., Driscoll, P. C., Keep, N. H., and Roumestand, C. (2004) *J Biomol NMR* **30**, 373-374
20. Canova, M. J., Kremer, L., and Molle, V. (2008) *Plasmid* **60**, 149-153
21. Barthe, P., Roumestand, C., Canova, M.J, Kremer, L., Hurard, C., Molle, V., and Cohen-Gonsaud, M. (2009) *Structure in press*
22. Sattler, M., Schleucher, J., and Griesinger, C. (1999) *Prog NMR Spectrosc* **34**, 93-158
23. Pons, J. L., Malliavin, T.E., and Delsuc, M.A. (1996) *J Biomol NMR* **8**, 445-452
24. Guntert, P. (2004) *Methods Mol Biol* **278**, 353-378
25. Cornilescu, G., Delaglio, F., and Bax, A. (1999) *J Biomol NMR* **13**, 289-302
26. Nederveen, A. J., Doreleijers, J. F., Vranken, W., Miller, Z., Spronk, C. A., Nabuurs, S. B., Guntert, P., Livny, M., Markley, J. L., Nilges, M., Ulrich, E. L., Kaptein, R., and Bonvin, A. M. (2005) *Proteins* **59**, 662-672
27. Laskowski, R. A., Moss, D. S., and Thornton, J. M. (1993) *J Mol Biol* **231**, 1049-1067
28. Koradi, R., Billeter, M., and Wuthrich, K. (1996) *J Mol Graph* **14**, 51-55, 29-32
29. Fiuza, M., Canova, M. J., Zanella-Cleon, I., Becchi, M., Cozzzone, A. J., Mateos, L. M., Kremer, L., Gil, J. A., and Molle, V. (2008) *J Biol Chem* **283**, 18099-18112
30. Aravind, L., Anantharaman, V., Balaji, S., Babu, M. M., and Iyer, L. M. (2005) *FEMS Microbiol Rev* **29**, 231-262
31. Sam, M. D., Cascio, D., Johnson, R. C., and Clubb, R. T. (2004) *J Mol Biol* **338**, 229-240
32. Cottin, V., Van Linden, A., and Riches, D. W. (1999) *J Biol Chem* **274**, 32975-32987
33. Garvie, C. W., and Wolberger, C. (2001) *Mol Cell* **8**, 937-946
34. Kang, C. M., Abbott, D. W., Park, S. T., Dascher, C. C., Cantley, L. C., and Husson, R. N. (2005) *Genes Dev* **19**, 1692-1704
35. Molle, V., Kremer, L., Girard-Blanc, C., Besra, G. S., Cozzzone, A. J., and Prost, J. F. (2003) *Biochemistry* **42**, 15300-15309
36. Alderwick, L. J., Molle, V., Kremer, L., Cozzzone, A. J., Dafforn, T. R., Besra, G. S., and Futterer, K. (2006) *Proc Natl Acad Sci U S A* **103**, 2558-2563
37. Sharma, K., Gupta, M., Pathak, M., Gupta, N., Koul, A., Sarangi, S., Baweja, R., and Singh, Y. (2006) *J Bacteriol* **188**, 2936-2944
38. Kumar, P., Kumar, D., Parikh, A., Rananaware, D., Gupta, M., Singh, Y., and Nandicoori, V. K. (2009) *J Biol Chem in press*

FOOTNOTES

¹**Abbreviations used in the text:** EMSA, electrophoretic mobility shift assay; *M. tb*, *M. tuberculosis*; STPK, Ser/Thr Protein Kinase.

NMR experiments were recorded and analysed using the facilities of the Structural Biology platform RIO (CBS, Montpellier, France). We thank Dr. Emmanuel Margeat for his help with fluorescence experiments, as well as Dr. Gilles Labesse and Jean-Luc Pons for their helpful discussions. The authors wish also to thank M. Becchi, I. Zanella-Cléon and A. Cornut (IBCP, Lyon, France) for their excellent expertise and technical assistance in mass spectrometry analysis.

This work was supported by the "Agence Nationale de la Recherche" (grant JC07_203251 for M. C-G, and ANR-06-MIME-027-01 for V.M. and L.K.).

FIGURE LEGENDS

Figure 1. Rv2175c NMR fingerprint. [^1H - ^{15}N] HSQC spectrum of Rv2175c from *M. tb* recorded at 30°C on a ^{15}N -uniformly labelled sample. Cross peak assignments are indicated using the one-letter amino acid and number.

Figure 2. Multiple sequence alignment of Rv2175c ortholog proteins from corynebacteria and mycobacteria. Sequence alignment of the *Mycobacterium tuberculosis* Rv2175c homologues in *Mycobacterium bovis*, *Mycobacterium avium paratuberculosis*, *Mycobacterium marinum*, *Mycobacterium leprae*, *Mycobacterium avium*, *Mycobacterium smegmatis*, *Mycobacterium abscessus*, *Corynebacterium glutamicum*, *Corynebacterium diphtheriae*, and *Streptomyces coelicolor*. Protein secondary element assignment for Rv2175c are represented on the top of the sequences. Numbering of amino acids corresponds to the Rv2175c protein from *M. tuberculosis*

Figure 3. Overall structure of Rv2175c. (A and B) Two views related by a 90° rotation along the z-axis of a cartoon representation of the Rv2175c structure. The residues Met1 to Ile16 and Thr110 to Asn122 are fully disordered and represented in green. **(C)** Closed-up of the internal face of the $\alpha 1$ and $\alpha 2$ helices. The N-terminal domain harbours an unusual prokaryotic winged Helix-Turn-Helix (wHTH) DNA-binding motif missing the typical third helix. The turn after the second strand (Ile66 and Phe71) caps the hydrophobic core formed by the two first helices of the wHTH, a role usually devoted to the third helix of the motif.

Figure 4. Conformational flexibility in Rv2175c. Representation of the final ensemble comprising 30 NMR structures of Rv2175c. The N-terminal wHTH domain is represented in red while the putative C-terminal effector domain is represented in grey. The residues Met1 to Pro17 and Asp106 to Ala123 are not represented. The superimposition made on the sole wHTH domain domain **(A)** and the C-terminal domain **(B)** shows that some flexibility existing between the two domains.

Figure 5. DNA-binding activity of Rv2175c. (A and B) Gel electrophoretic mobility shift assays of the binding of Rv2175c to a non-relevant 200 bp DNA fragment. The radioactive 200 bp DNA probe was incubated with 0.4, 0.6, 0.8 and 1 μg of the purified Rv2175c, Rv2175c_T9D, or phosphorylated Rv2175c by PknL (pT9-Rv2175c) and analysed by non-denaturing PAGE. As a control, non radiolabeled DNA (cold probe) was added to the Rv2175 mix. **(C and D)** Anisotropy binding profile for binding of Rv2175c **(B)** and Rv2175c_T9D **(C)** to a 23-bp oligonucleotide labeled with Alexa Fluor 488 succinimidyl ester dye. The protein concentration was 4 nM. The buffer was 10 mM Tris-HCl, pH 7.5 with 0mM NaCl (\square), 50mM NaCl (\circ), 100mM (\times) or 150mM NaCl (\blacktriangle). The anisotropy measurements were performed four different times and showed consistent order of magnitude.

Figure 6. Identification of Rv2175c phosphorylation site. (A) MS/MS spectrum of the doubly charged ion $[\text{M}+2\text{H}]^{2+}$ at m/z 426.7 of peptide [5-12] (monoisotopic mass : 851.40 Da). Unambiguous location of the phosphate group on T9 was showed by observation of the “y” C-terminal daughter ion series. Starting from the C-terminal residue, all “y” ions loose phosphoric acid (-98 Da) after the T9 phosphorylated residue. **(B)** *In vitro* phosphorylation of the Rv2175c_T9A mutant. Purified Rv2175c and Rv2175c_T9A were incubated with PknL₁₋₂₈₁ and [γ - ^{32}P]ATP. Samples were separated by SDS-PAGE (upper panel) and visualized by autoradiography (lower panel).

Figure 7. Chemical shift perturbations following Rv2175c phosphorylation. Amide averaged chemical shift differences ($\Delta\delta$) as a function of the protein sequence. $\Delta\delta$ have been calculated between the amide group resonances of Rv2175c non-phosphorylated and Rv2175c phosphorylated by PknL (pRv2175c) using $\Delta\delta = [\Delta\delta_{\text{H}^2} + (\Delta\delta_{\text{N},\gamma_{\text{N}}/\gamma_{\text{H}}})^2]^{0.5}$ at pH 4.6 **(A)** and pH 6.8 **(C)**. [^1H - ^{15}N] HSQC spectrum superimposition of Rv2175c non-phosphorylated (in black) with pRv2175c (in red) at pH 4.6. The Thr9 and the phosphorylated Thr9 (pThr) resonances are squared in black and red respectively **(B)**. [^1H - ^{15}N] HSQC spectrum superimposition of pRv2175c at pH 4.6 (in red), pH 5.0 (in yellow), pH 5.3 (in light green), pH 6.1 (in dark green), pH 6.4 (in blue), pH 6.8 (in purple). The pThr resonance is squared in the corresponding colours **(D)**.

Table 1. NMR and refinement statistics for Rv2175c protein structures

	Protein
NMR distance and dihedral constraints	
Distance constraints	
Total NOE	1676
Intra-residue	477
Inter-residue	
Sequential ($ i-j = 1$)	576
Medium-range ($ i-j < 4$)	278
Long-range ($ i-j > 5$)	345
Intermolecular	
Hydrogen bonds	66
Total dihedral angle restraints	
ϕ	95
ψ	95
χ_1	17
Structure statistics	
Violations (mean and s.d.)	
Max. distance constraint violation (Å)	0.16 ± 0.02
Max. dihedral angle violation (°)	4.00 ± 0.88
Deviations from idealized geometry	
Bond lengths (Å)	0.0095 ± 0.0002
Bond angles (°)	1.1437 ± 0.0241
Improper (°)	1.2872 ± 0.0643
Ramachandran plot (%)	
Most favoured region	93.2
Additionally allowed region	6.4
Generously allowed region	0.3
Disallowed region	0.1
Average pairwise r.m.s. deviation* (Å)	
Backbone	0.87 ± 0.15
Heavy	1.54 ± 0.18

* "Pairwise r.m.s. deviation was calculated among 30 refined structures for residues 18-104,126-146."]

Figure 2

Downloaded from www.jbc.org/

M.tubMPGRAPGSTLARVGSIPAGDDVLDPPDEPTYDLPRAV¹AE²LL³GV⁴PV⁵SK⁶V⁷A⁸Q⁹LR¹⁰EG¹¹CH¹²LV¹³AV¹⁴RR¹⁵AG¹⁶GV¹⁷IP¹⁸QV¹⁹FF²⁰T
 M.bovMPGRAPGSTLARVGSILAGDDVLDPPDEPTYDLPRAV¹AE²LL³GV⁴PV⁵SK⁶V⁷A⁸Q⁹LR¹⁰EG¹¹CH¹²LV¹³AV¹⁴RR¹⁵AG¹⁶GV¹⁷IP¹⁸QV¹⁹FF²⁰T
 M.avi_{par} MVIGRRNTGSTRASIFLARVSSIPAGDDVLDPPDEPTYDLPRAV¹AE²LL³GV⁴PV⁵SK⁶V⁷A⁸Q⁹LR¹⁰EG¹¹CH¹²LV¹³AV¹⁴RR¹⁵AG¹⁶GV¹⁷IP¹⁸QV¹⁹FF²⁰T
 M.marMGSIPAGDDVLDPPDEPTYDLPRAV¹AE²LL³GV⁴PV⁵SK⁶V⁷A⁸Q⁹LR¹⁰EG¹¹CH¹²LV¹³AV¹⁴RR¹⁵AG¹⁶GV¹⁷IP¹⁸QV¹⁹FF²⁰T
 M.lepMGSIPAGDDVLDPPDEPTYDLPRAV¹AE²LL³GV⁴PV⁵SK⁶V⁷A⁸Q⁹LR¹⁰EG¹¹CH¹²LV¹³AV¹⁴RR¹⁵AG¹⁶GV¹⁷IP¹⁸QV¹⁹FF²⁰T
 M.aviMSSIPAGDDVLDPPDEPTYDLPRAV¹AE²LL³GV⁴PV⁵SK⁶V⁷A⁸Q⁹LR¹⁰EG¹¹CH¹²LV¹³AV¹⁴RR¹⁵AG¹⁶GV¹⁷IP¹⁸QV¹⁹FF²⁰T
 M.smeMSSIPAADVLDPPDEAVYDLPRAV¹AE²LL³GV⁴PV⁵SK⁶V⁷A⁸Q⁹LR¹⁰EG¹¹CH¹²LV¹³AV¹⁴RR¹⁵AG¹⁶GV¹⁷IP¹⁸QV¹⁹FF²⁰T
 M.absMSAIPYVADVLDADPELFTLKEV¹AR²LV³PK⁴Q⁵YL⁶RD⁷GE⁸LI⁹AV¹⁰KG¹¹GG¹²EL¹³KV¹⁴VP¹⁵VI¹⁶FF¹⁷S
 C.gluMSSNNESSALPNDPELFTLKEV¹AR²LV³PK⁴Q⁵YL⁶RD⁷GE⁸LI⁹AV¹⁰KG¹¹GG¹²EL¹³KV¹⁴VP¹⁵VI¹⁶FF¹⁷S
 C.dipMSNNIVPSSVLPAGENLLTVPD¹AE²RI³G⁴Q⁵VP⁶TR⁷FD⁸Q⁹I¹⁰GE¹¹KK¹²LI¹³AV¹⁴KG¹⁵GG¹⁶EL¹⁷KV¹⁸VP¹⁹VI²⁰FF²¹S
 S_coeMTEIDAKTDLVPP¹..²AWL³TV⁴PP⁵IA⁶EML⁷GV⁸EV⁹TR¹⁰Q¹¹LI¹²NE¹³GO¹⁴LI¹⁵AV¹⁶RR¹⁷GEN¹⁸ER¹⁹AL²⁰TH²¹IP²²AA²³LF²⁴

M.tubNSCGVVK¹SL²LP³GL⁴LT⁵LD⁶HD⁷GG⁸X⁹RD¹⁰TE¹¹IM¹²EW¹³LF¹⁴TP¹⁵DF¹⁶SL¹⁷TI¹⁸TR¹⁹DG²⁰SR²¹DA²²V²³SN²⁴AR²⁵VD²⁶AL²⁷HA²⁸HH²⁹Q³⁰ARE³¹V³²RR³³AA³⁴Q³⁵AA³⁶Y³⁷
 M.bovNSCGVVK¹SL²LP³GL⁴LT⁵LD⁶HD⁷GG⁸X⁹RD¹⁰TE¹¹IM¹²EW¹³LF¹⁴TP¹⁵DF¹⁶SL¹⁷TI¹⁸TR¹⁹DG²⁰SR²¹DA²²V²³SN²⁴AR²⁵VD²⁶AL²⁷HA²⁸HH²⁹Q³⁰ARE³¹V³²RR³³AA³⁴Q³⁵AA³⁶Y³⁷
 M.avi_{par}KSGEVVK¹SL²LP³GL⁴LT⁵LD⁶HD⁷GG⁸X⁹RD¹⁰TE¹¹IV¹²RW¹³LF¹⁴TP¹⁵DF¹⁶SL¹⁷TV¹⁸TR¹⁹DG²⁰SR²¹DA²²V²³SN²⁴AR²⁵VD²⁶AL²⁷HA²⁸HH²⁹Q³⁰ARE³¹V³²RR³³AA³⁴Q³⁵AA³⁶Y³⁷
 M.marSSGQVVK¹SL²LP³GL⁴LT⁵LD⁶HD⁷GG⁸X⁹RD¹⁰TE¹¹IV¹²RW¹³LF¹⁴TP¹⁵DF¹⁶SL¹⁷TV¹⁸TR¹⁹DG²⁰SR²¹DA²²V²³SN²⁴AR²⁵VD²⁶AL²⁷HA²⁸HH²⁹Q³⁰ARE³¹V³²RR³³AA³⁴Q³⁵AA³⁶Y³⁷
 M.lepNSGAVVK¹SL²LP³GL⁴LT⁵LD⁶HD⁷GS⁸FF⁹HT¹⁰E¹¹IV¹²RW¹³LF¹⁴TP¹⁵DF¹⁶SL¹⁷TL¹⁸TR¹⁹DG²⁰SR²¹DA²²V²³SN²⁴AR²⁵VD²⁶AL²⁷HA²⁸HH²⁹Q³⁰ARE³¹V³²RR³³AA³⁴Q³⁵AA³⁶Y³⁷
 M.aviKSGEVVK¹SL²LP³GL⁴LT⁵LD⁶HD⁷GG⁸X⁹RD¹⁰TE¹¹IV¹²RW¹³LF¹⁴TP¹⁵DF¹⁶SL¹⁷TV¹⁸TR¹⁹DG²⁰SR²¹DA²²V²³SN²⁴AR²⁵VD²⁶AL²⁷HA²⁸HH²⁹Q³⁰ARE³¹V³²RR³³AA³⁴Q³⁵AA³⁶Y³⁷
 M.smeDAGHVV¹KS²LP³GL⁴LV⁵VR⁶RD⁷GG⁸X⁹HT¹⁰NE¹¹IL¹²RW¹³LF¹⁴TP¹⁵DF¹⁶SL¹⁷TI¹⁸TR¹⁹DG²⁰SR²¹DA²²V²³SN²⁴AR²⁵VD²⁶AL²⁷HA²⁸HH²⁹Q³⁰ARE³¹V³²RR³³AA³⁴Q³⁵AA³⁶Y³⁷
 M.absPEGFVV¹KH²LF³GL⁴LV⁵VR⁶RD⁷GG⁸X⁹HE¹⁰PE¹¹IL¹²RW¹³LF¹⁴AG¹⁵DE¹⁶SL¹⁷TV¹⁸SR¹⁹DG²⁰TT²¹ER²²IE²³EA²⁴RE²⁵VD²⁶AL²⁷HA²⁸HH²⁹Q³⁰ARE³¹V³²RR³³AA³⁴Q³⁵AA³⁶Y³⁷
 C.gluTKKEN¹TNR²FI³GV⁴IAL⁵FD⁶GG⁷SD⁸EE⁹IL¹⁰AF¹¹LF¹²TE¹³DE¹⁴SL¹⁵LP¹⁶.....GR¹⁷ED¹⁸AL¹⁹H²⁰CG²¹L²²ARE²³V²⁴RR²⁵AA²⁶Q²⁷AA²⁸Y²⁹
 C.dipEKGT¹NK²FV³GV⁴IAL⁵FD⁶GG⁷XT⁸DO⁹E¹⁰IF¹¹MF¹²LF¹³TE¹⁴DE¹⁵SL¹⁶LP¹⁷.....GR¹⁸ED¹⁹AI²⁰H²¹CG²²L²³ARE²⁴V²⁵RR²⁶AA²⁷Q²⁸AA²⁹Y³⁰
 S_coeIDED¹KV²VK³GL⁴GL⁵TL⁶TL⁷RD⁸DG⁹FD¹⁰DE¹¹ML¹²EW¹³LF¹⁴TP¹⁵DF¹⁶SL¹⁷LP¹⁸.....GT¹⁹EA²⁰AL²¹SN²²KG²³TE²⁴V²⁵RR²⁶AA²⁷Q²⁸AA²⁹Y³⁰

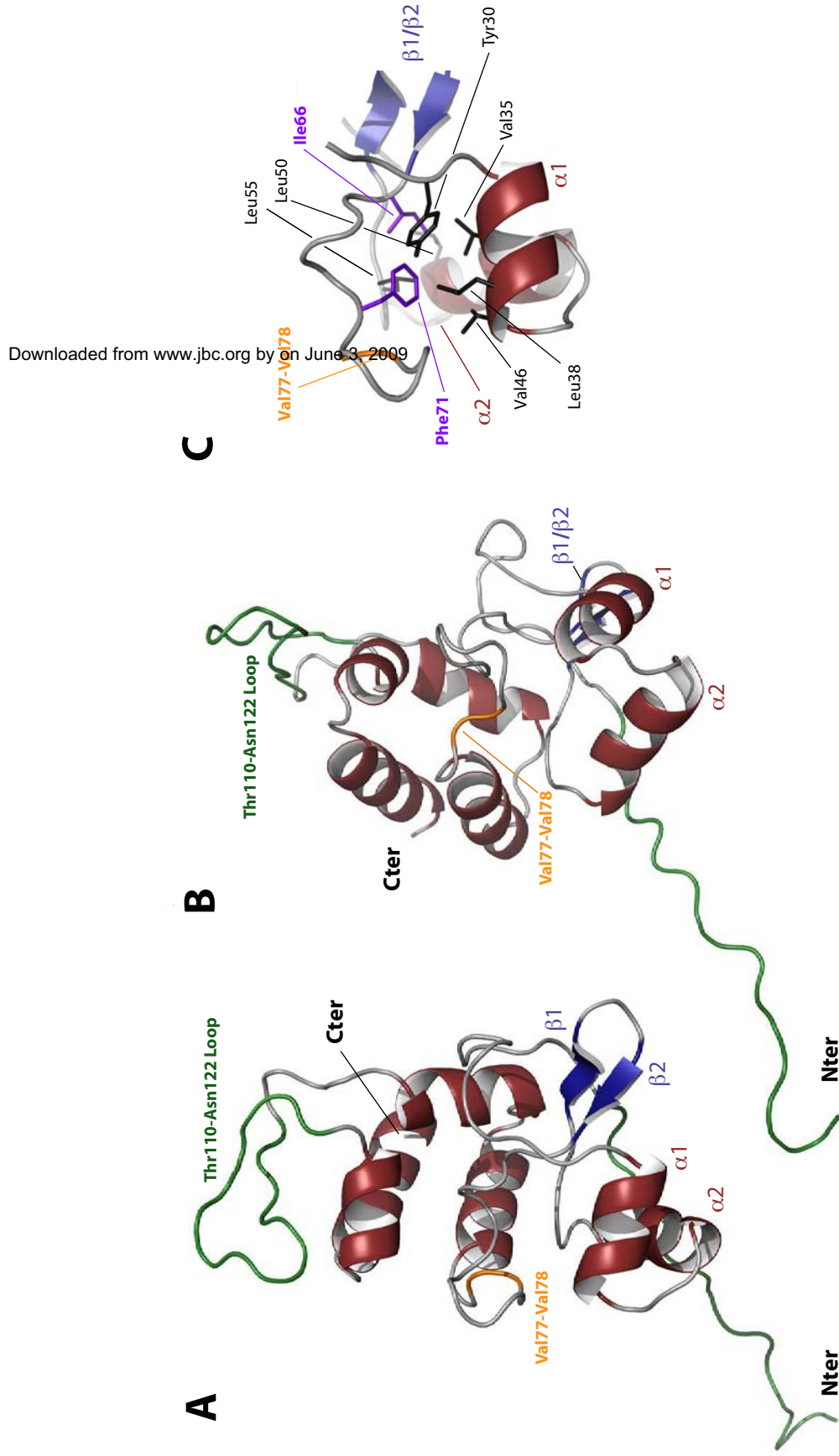
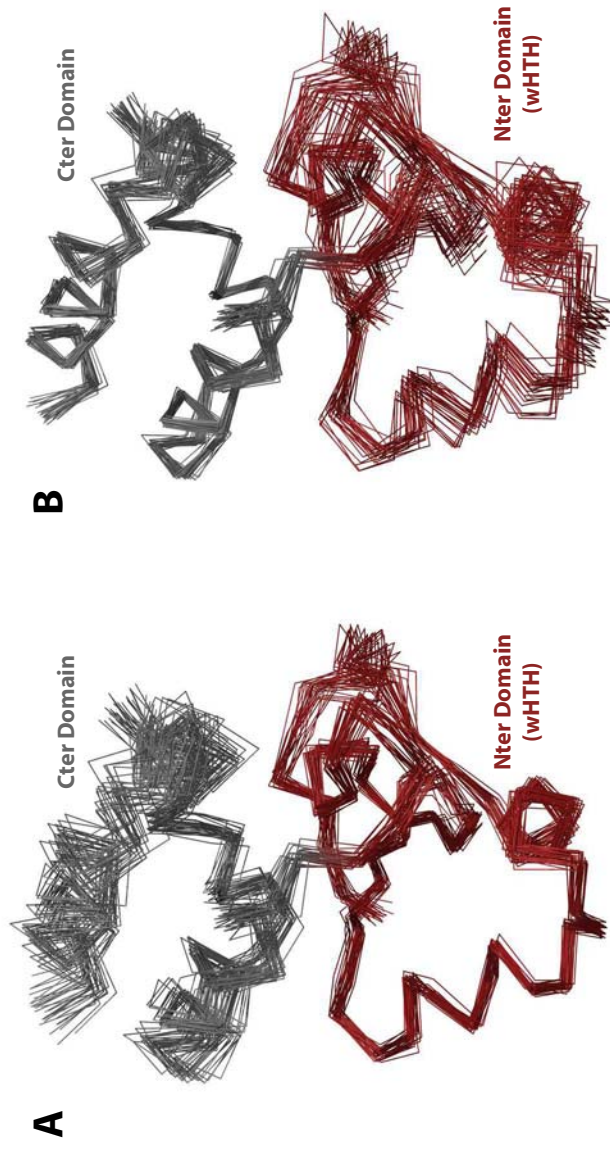
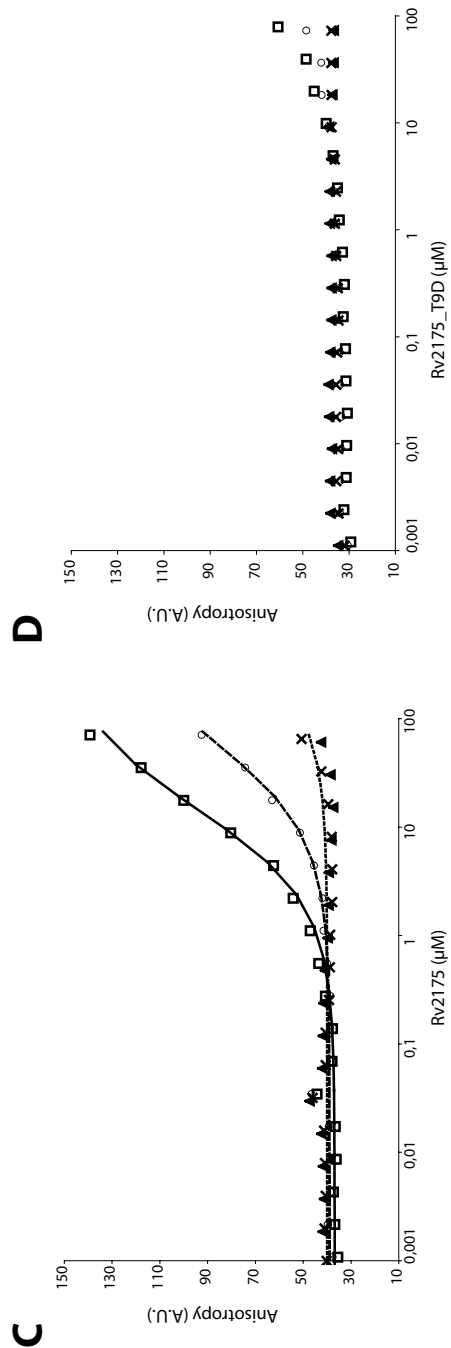
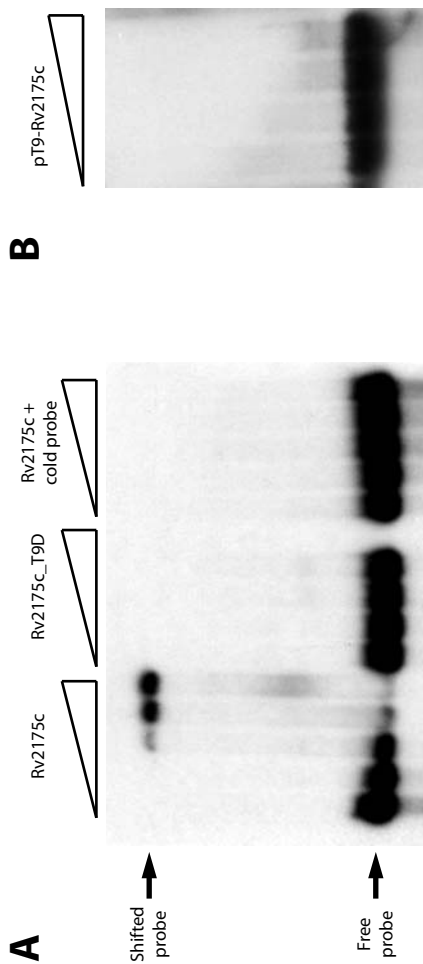


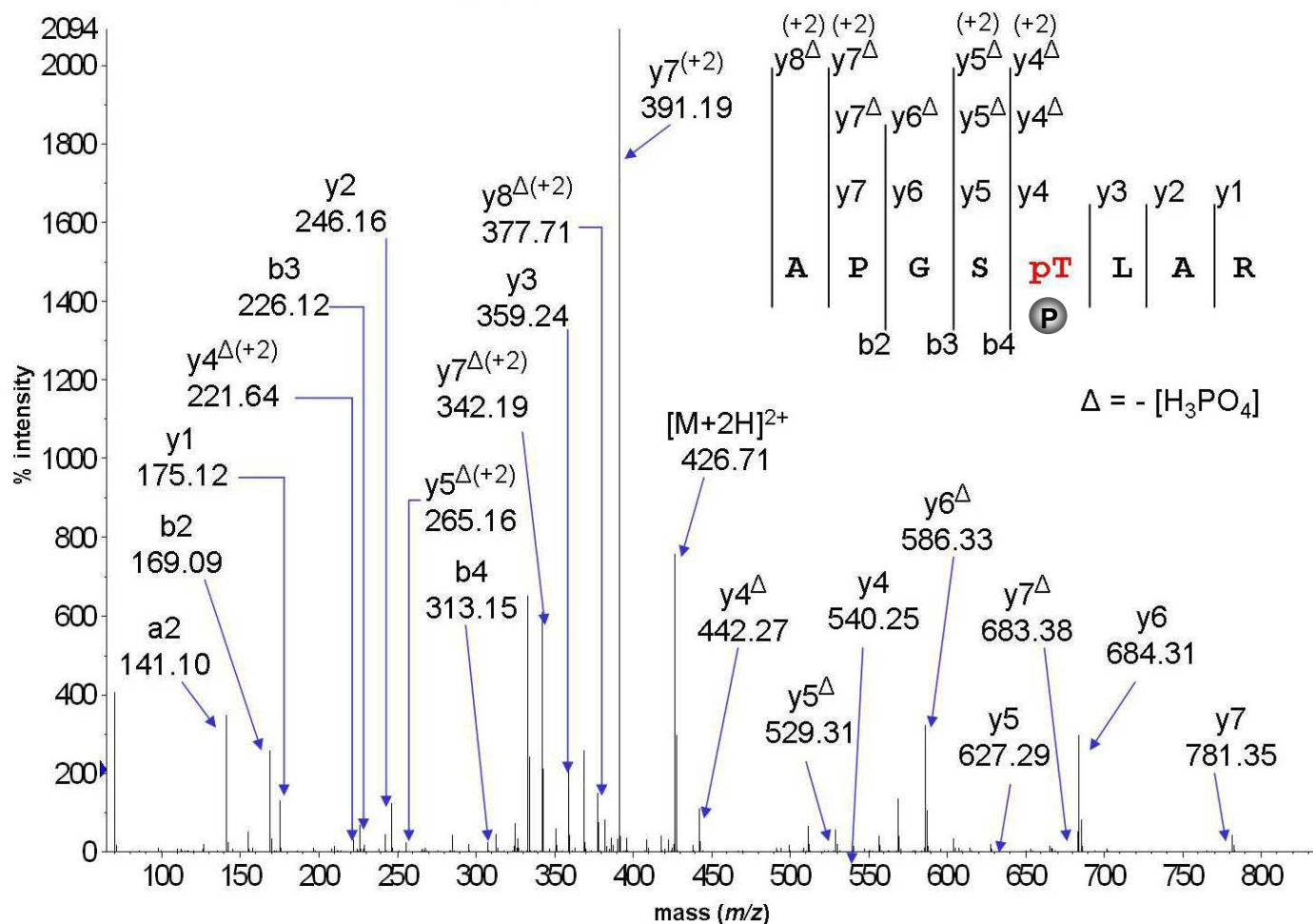
Figure 4



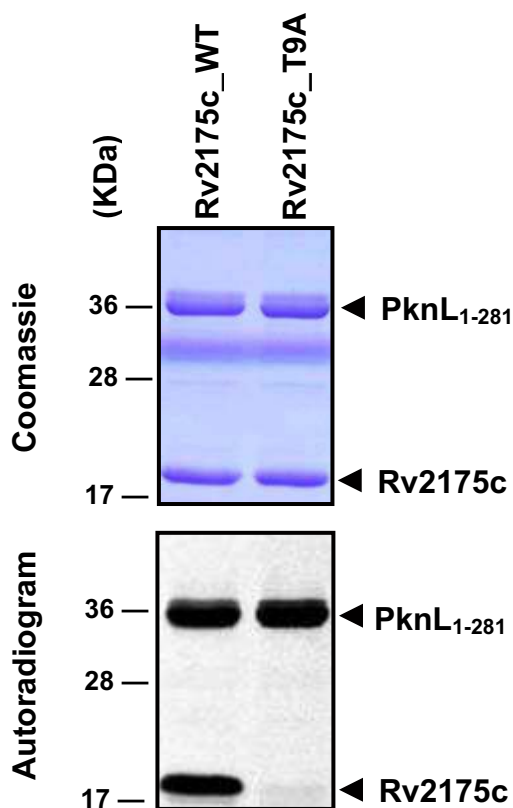
Downloaded from www.jbc.org by on June 3, 2009

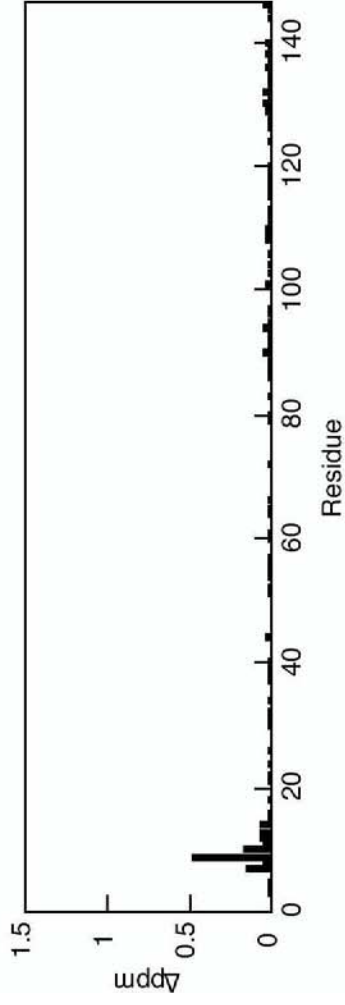
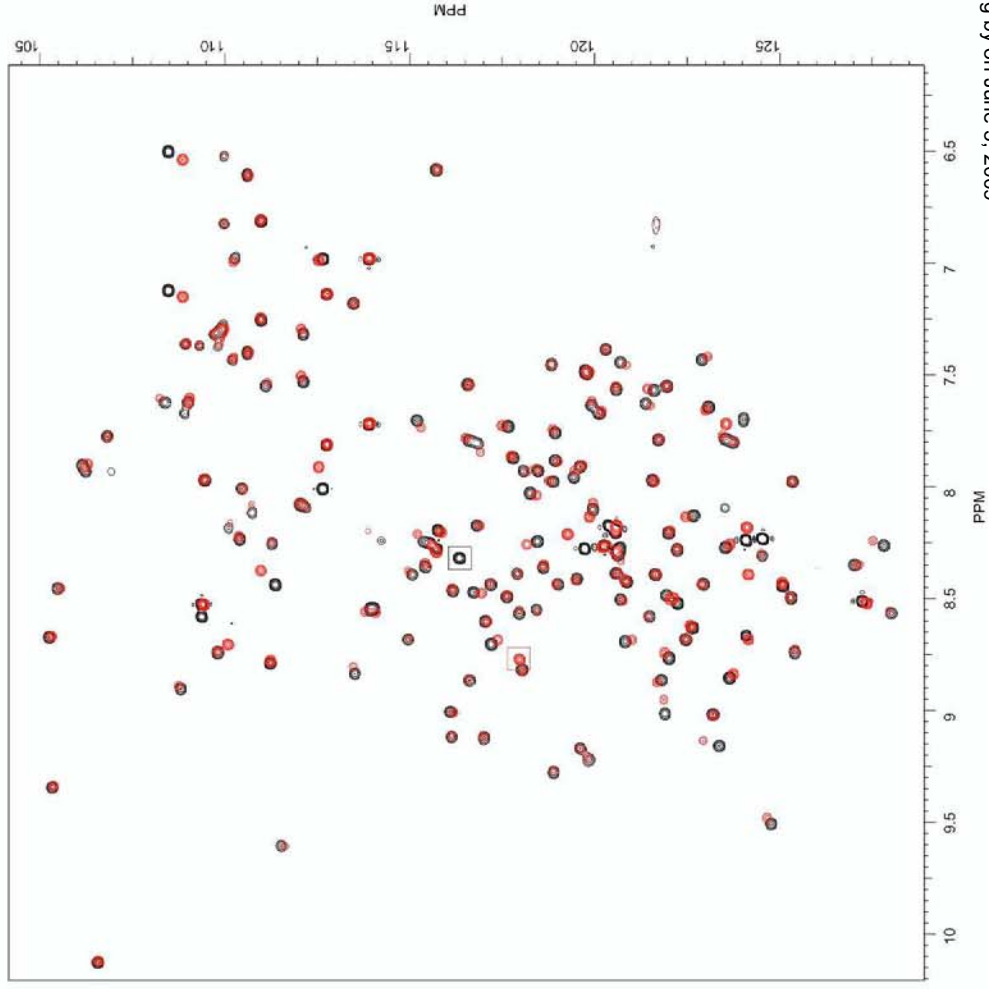
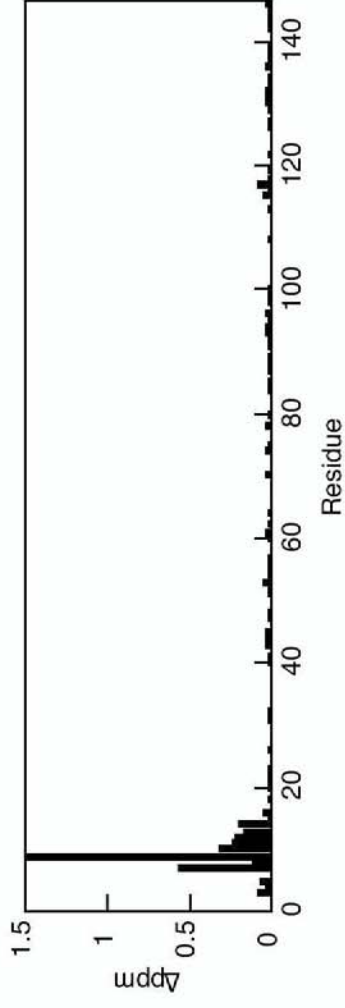
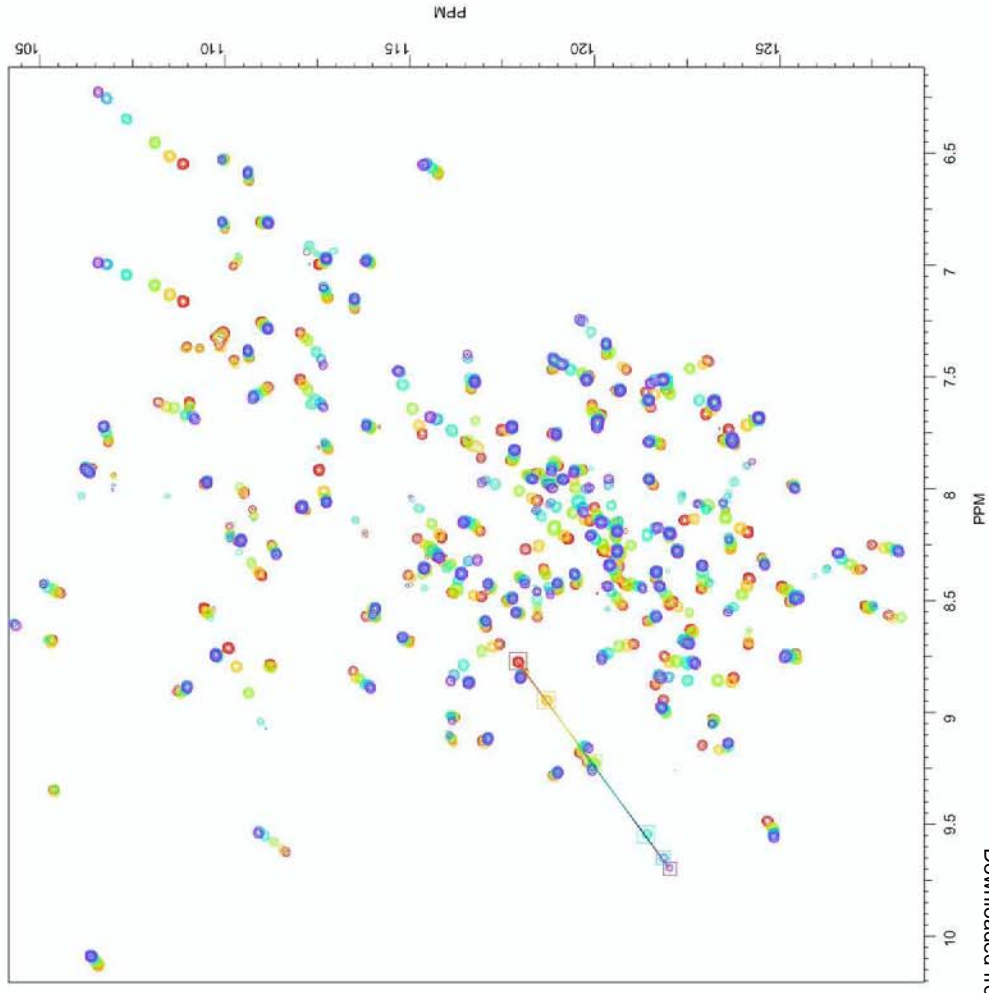


A



B



A**B****C****D**

Article III

**pETPhos: a customized expression vector designed for further
characterization of Ser/Thr/Tyr protein kinases and their substrates**

Canova MJ, Kremer L, and Molle V

Plasmid. 2008, 60:149-53

Présentation de l'article III :

La détection et l'identification des mécanismes biochimiques et physiologiques régulés via le processus de phosphorylation sont limitées par le manque d'outils de biologie moléculaire dédiés à l'étude de ces protéines phosphorylées.

En effet, une des limitations de ces études est due à la présence de résidus séryles et thréonyles sur les étiquettes 6-His des vecteurs de surexpression classiques de type pET ou autres utilisés lors de la purification des protéines recombinantes. Ces résidus (Ser et Thr) peuvent en effet se retrouver phosphorylés de manière non spécifique et, par conséquent, générer de faux-positifs ne correspondant pas à de "réels" substrats des kinases testées. Suite aux problèmes de phosphorylation non-spécifique rencontrés lors de la caractérisation du couple PknL/Rv2175c, nous avons construit le vecteur de surexpression pETPhos, un vecteur d'expression dérivé du vecteur pETTEv, permettant la production de protéines recombinantes possédant une étiquette 6-His, et également possédant un site de coupure à la protéase TEV (Tobacco Etch Virus).

L'intérêt de l'utilisation de ce nouveau vecteur, pETPhos, est illustré dans cet article par le biais d'une étude comparative de la phosphorylation de la protéine Rv2175c exprimée soit dans un vecteur classique pET de surexpression (Novagen), soit avec le nouveau vecteur pETPhos. En effet, l'analyse des aminoacides phosphorylés par PknL *in vitro* permet de détecter la présence de sérine(s) et thréonine(s) phosphorylées lorsque Rv2175c est exprimée *via* le vecteur pET classique, alors que seuls des résidus thréonyles sont révélés lorsque Rv2175c est exprimée avec le vecteur pETPhos. Ces résultats montrent clairement que les STPKs, en l'occurrence PknL lors de cette étude, sont capables de phosphoryler des résidus présents au niveau de l'étiquette 6-His des protéines recombinantes et, par conséquent, de générer de faux positifs et conduire à des interprétations erronées des tests de phosphorylation. Ce vecteur représente donc un outil particulièrement intéressant pour la recherche et l'identification de nouveaux substrats ainsi que pour la caractérisation des sites de phosphorylation sans ambiguïté.

The *Mycobacterium tuberculosis* GroEL1 Chaperone Is a Substrate of Ser/Thr Protein Kinases[∇]

Marc J. Canova,¹ Laurent Kremer,^{2,3*} and Virginie Molle^{1*}

Institut de Biologie et Chimie des Protéines (IBCP UMR 5086), CNRS, Université Lyon 1, IFR128 BioSciences, Lyon-Gerland, 7 passage du Vercors, 69367 Lyon Cedex 07, France¹; Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, Universités de Montpellier II et I, CNRS 5235, case 107, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France²; and INSERM, DIMNP, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France³

Received 4 November 2008/Accepted 30 January 2009

We demonstrate that *Mycobacterium tuberculosis* GroEL1 is phosphorylated by PknF at two positions, Thr25 and Thr54. Unexpectedly, *Mycobacterium smegmatis* GroEL1 is not a substrate of its cognate PknF. This study shows that the phosphorylation profiles of conserved proteins are species dependent and provide insights that may explain the numerous biological functions of these important proteins.

Heat shock protein 60 (Hsp60), also known as Cpn60 or GroEL, is a prototypical molecular chaperone, which in *Escherichia coli* has been shown to be required for growth and necessary for the folding of several essential proteins (11, 18). Although most bacteria possess a single copy of the *groEL* gene (22), actinomycetes are unusual in having genes that encode two forms of the Hsp60 chaperone GroEL. The major human pathogen *Mycobacterium tuberculosis* GroEL1 and GroEL2 proteins (20) share 61% amino acid identity. In *M. tuberculosis*, both GroEL proteins are upregulated during heat shock (32), oxidative stress response (9), and macrophage infection (27). Moreover, several studies reported their involvement in the immune response to *M. tuberculosis* infection (21, 29). However, *M. tuberculosis* GroEL proteins are unusual chaperones in the sense that they exhibit very weak ATPase activity and they are poorly active in refolding substrates. Another intriguing feature of the *M. tuberculosis* GroEL proteins is the absence of the canonical oligomeric state, suggesting that the mycobacterial chaperones have a completely different folding mechanism than that of the prototypic GroEL (31). This raises questions about the nature of the biological functions of these chaperones and whether they are required for intracellular protein folding or associated with virulence in the infected host (30). Whereas *groEL2* was found to be essential for cell survival, a *groEL1* mutant was indistinguishable from the wild-type strain either in broth culture or within macrophages. However, infection studies in mice indicated the failure of the *groEL1* mutant to produce a granulomatous inflammation ei-

ther in mice or guinea pigs, which correlated with a reduced cytokine expression (15). Thus, GroEL1 appears to be a key factor in controlling the cytokine-dependent granulomatous response during *M. tuberculosis* infection. However, whether this immunomodulatory function represents the primary role of GroEL1 in mycobacteria remains uncertain, as the protein is also present in atypical and nonpathogenic species, such as *Mycobacterium smegmatis*. In this species, *groEL1* contains the *attB* site for phage Bxb1 integration (19) and was found to be dispensable for normal planktonic growth (28). The unexpected role of GroEL1 in biofilm formation by physically interacting with the β -ketoacyl-AcpM synthase KasA, a key component of the type II fatty acid synthase involved in mycolic acid biosynthesis, has recently been reported (2, 28). In general, different GroEL1 proteins exhibit a bewildering variety of biological functions, and albeit the *M. tuberculosis* and *M. smegmatis* GroEL1 orthologues display very highly conserved sequences, they do not behave similarly. The extensive literature on GroEL1 proteins seems to suggest that these molecules are not unitary and that different GroEL1 proteins from different sources could express different patterns of biological activity. It appears very likely that slight changes in the sequence and/or structure of mycobacterial GroEL proteins can exert profound effects on their biological activity. Although the biochemical, functional, and structural properties of mycobacterial GroEL1 proteins have been studied (30), it is not known whether these proteins undergo posttranslational modifications. Therefore, as a first step to decipher how subtle primary sequence changes may confer different activities to the *M. tuberculosis* and *M. smegmatis* GroEL1 proteins, we investigated whether these proteins exhibit differential posttranslational modifications.

STPK-mediated phosphorylation of *M. tuberculosis* GroEL1. We first investigated whether the *M. tuberculosis* GroEL1 chaperone could be modified by phosphorylation, which would provide valuable insights into some of their functional properties. Protein phosphorylation allows extracellular signals to be translated in cellular responses and has recently emerged as a major physiological mechanism due to its link to virulence in different pathogens (14, 35). The *M. tuberculosis* genome en-

* Corresponding author. Mailing address for Virginie Molle: Institut de Biologie et Chimie des Protéines (IBCP UMR 5086), CNRS, Université Lyon 1, IFR128 BioSciences, Lyon-Gerland, 7 passage du Vercors, 69367 Lyon Cedex 07, France. Phone: (33) 4 72 72 26 79. Fax: (33) 4 72 72 26 41. E-mail: vmolle@ibcp.fr. Mailing address for Laurent Kremer: Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, Universités de Montpellier II et I, CNRS 5235, case 107, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France. Phone: (33) 4 67 14 33 81. Fax: (33) 4 67 14 42 86. E-mail: laurent.kremer@univ-montp2.fr.

[∇] Published ahead of print on 6 February 2009.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and use or description	Source or reference
Strains		
<i>E. coli</i> TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) ϕ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697	Invitrogen
<i>E. coli</i> BL21(DE3)Star	<i>galU galK rpsL endA1 nupG</i> ; used for general cloning F ⁻ <i>ompT hsdSB</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3); used to express recombinant proteins in <i>E. coli</i>	Stratagene
Plasmids		
pETPhos	pET15b (Novagen) derivative including the replacement of the thrombin site coding sequence with a tobacco etch virus protease site and Ser-to-Gly mutagenesis in the N-terminal His tag	4
pETPhos_ <i>groEL1</i>	pETPhos derivative used to express His-tagged fusion of GroEL1	This work
pETPhos_ <i>groEL1_T25A</i>	pETPhos derivative used to express His-tagged fusion of GroEL1(T25A)	This work
pETPhos_ <i>groEL1_T54A</i>	pETPhos derivative used to express His-tagged fusion of GroEL1(T54A)	This work
pETPhos_ <i>groEL1_T25A/T54A</i>	pETPhos derivative used to express His-tagged fusion of GroEL1(T25A/T54A)	This work
pETPhos_ <i>pknF</i>	pETPhos derivative used to express His-tagged fusion of the PknF cytosolic domain	This work
pETPhos_ <i>pknF_K41M</i>	pETPhos derivative used to express His-tagged fusion of the PknF(K41M) cytosolic domain	This work
pETPhos_ <i>pknF_T173A</i>	pETPhos derivative used to express His-tagged fusion of the PknF(T173A) cytosolic domain	This work
pETPhos_ <i>pknF_T175A</i>	pETPhos derivative used to express His-tagged fusion of the PknF(T175A) cytosolic domain	This work
pETPhos_ <i>pknF_T173A/T175A</i>	pETPhos derivative used to express His-tagged fusion of the PknF(T173A/T175A) cytosolic domain	This work
pETPhos_ <i>pknF_Msm</i>	pETPhos derivative used to express His-tagged fusion of the PknF cytosolic domain from <i>M. smegmatis</i>	This work
pETPhos_ <i>groEL1_Msm_WT</i>	pETPhos derivative used to express His-tagged fusion of GroEL1(WT) from <i>M. smegmatis</i>	This work
pETPhos_ <i>groEL1_Msm_A25T</i>	pETPhos derivative used to express His-tagged fusion of GroEL1(A25T) from <i>M. smegmatis</i>	This work

codes 11 Ser/Thr protein kinases (STPKs) (1, 6), and most are being investigated for their physiological roles and potential application for future drug development to combat tuberculosis (36). The presence of several STPKs suggests that phosphorylation influences a wide range of biological functions, such as adaptation to various environmental conditions, stress, cell wall synthesis, cell division, and pathogenicity (36). Here, we tested the hypothesis whether *M. tuberculosis* STPKs could phosphorylate GroEL1. PknA, PknB, PknD, PknE, PknF, PknH, and PknL were expressed as His-tagged fusions and purified from *E. coli* as described previously (23). Recombinant GroEL1 was expressed and purified from *E. coli* BL21(DE3)Star harboring the pETPhos_GroEL1 plasmid (Table 1). When the STPKs were incubated in the presence of GroEL1 and [γ -³³P]ATP, phosphorylation of GroEL1 was observed with the different kinases, although the levels of the GroEL1 signal varied (Fig. 1). In fact, while all selected kinases, present at comparable concentrations, were able to phosphorylate GroEL1, PknF generated the strongest signal, thus leading to the hypothesis that GroEL1 could be preferentially recruited by PknF (Fig. 1). Together, these data indicate different levels of substrate specificity of the various STPKs, as reported previously for other mycobacterial substrates (23). A corollary arising from these experiments is that GroEL1 interacting with several STPKs may be regulated in vivo by multiple environmental signals, although this remains to be established.

Contribution of the activation loop Thr173 and Thr175 residues in the autophosphorylation activity of PknF. *M. tuberculosis* PknF participates in multiple biological processes, such as cell growth, septum formation, and glucose transport (8). It has been shown to phosphorylate the two Forkhead-associated domains of the ABC transporter protein Rv1747 (7, 26) and the Forkhead-associated domain of Rv0020 (13). The autokinase activity of PknF was demonstrated by incubating the protein with [γ -³³P]ATP. Wild-type PknF [PknF(WT)] incorpo-

rated radioactive phosphate from [γ -³³P]ATP, giving rise to a strong radioactive signal at the expected size of the protein, indicating that PknF undergoes autophosphorylation (Fig. 2A). To exclude the possibility of exogenous contamination that could explain labeling of PknF, Lys41 present in subdomain II of PknF was mutated to Met (Tables 1 and 2). Purified PknF(K41M) was incubated with [γ -³³P]ATP, and as expected, no signal could be detected, confirming the essential role of Lys41 in catalyzing the phosphorylation reaction, in agreement

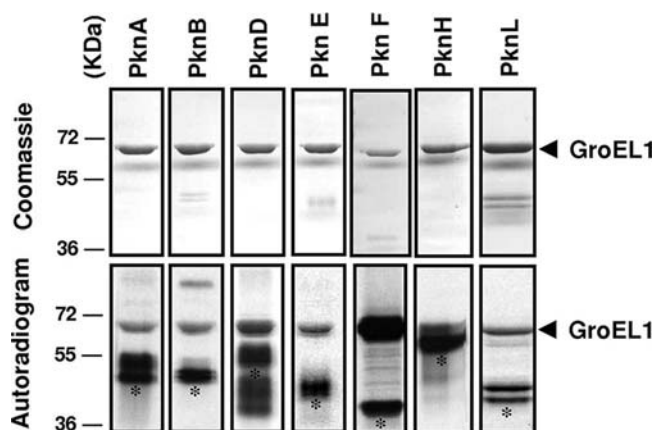


FIG. 1. In vitro phosphorylation of GroEL1 by STPKs. Seven recombinant STPKs (PknA, PknB, PknD, PknE, PknF, PknH, and PknL) encoded by the *M. tuberculosis* genome were expressed and purified as His-tagged fusions in *E. coli* and incubated with the purified His-tagged *M. tuberculosis* GroEL1. The different proteins were incubated together with [γ -³³P]ATP for 15 min, subjected to gel electrophoresis, and stained with Coomassie blue (top panel). Radioactive bands were revealed by autoradiography (bottom panel). Standard proteins of known molecular masses (in kilodaltons) were run in parallel, and their positions are shown to the left of the gels. Asterisks indicate the autophosphorylated kinases.

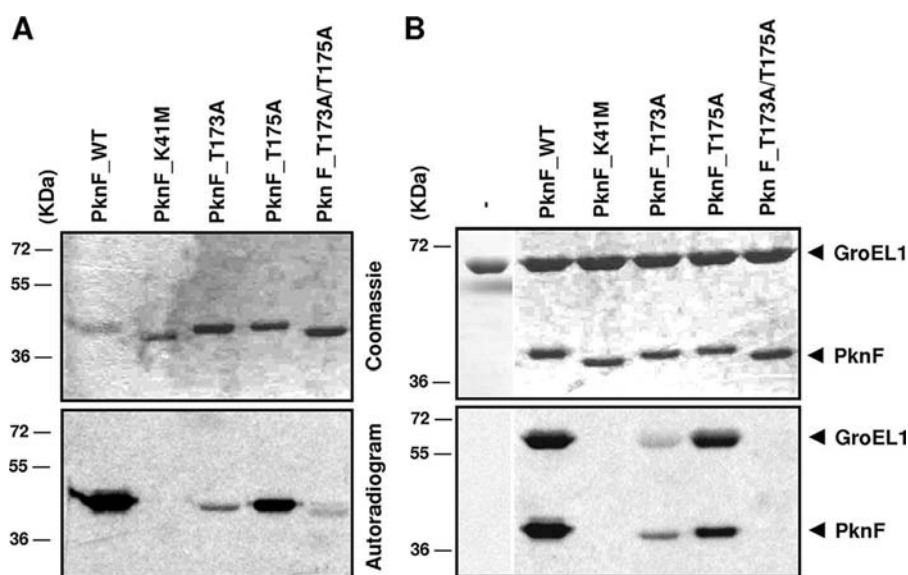


FIG. 2. In vitro phosphorylation of *M. tuberculosis* GroEL1 by PknF and the different PknF mutants. (A) Autokinase activity of the different derivatives of PknF. All proteins [PknF(WT), PknF(K41M), PknF(T173A), PknF(T175A), and PknF(T173A/T175A)] were overproduced in *E. coli*, purified as His-tagged fusions, and incubated with [γ - 33 P]ATP. Proteins were separated by SDS-PAGE and stained with Coomassie blue (top panel), and radioactive bands were revealed by autoradiography (bottom panel). (B) In vitro phosphorylation of GroEL1 by PknF and the different PknF mutants in the presence of [γ - 33 P]ATP. Proteins were separated by SDS-PAGE and stained with Coomassie blue (top panel), and radioactive bands were revealed by autoradiography (bottom panel). The positions of molecular mass markers (in kilodaltons) are shown to the left of the gels.

with previous reports on other mycobacterial kinases (24). The transition between active and inactive forms occurring via control of access to the catalytic site and/or substrate-binding site reflects one possible regulatory mechanism in eukaryote-type kinases. This involves phosphorylation/dephosphorylation steps through an autocatalytic mechanism or by intervention of other kinases and phosphatases. The active/inactive conformational switch is driven by the activation loop in numerous kinases (16), and the conformation of the activation loop depends on the phosphorylation state of this major control element (17). Boitel et al. (3) demonstrated that Thr171 and Thr173 are essential phosphoacceptors in *M. tuberculosis* PknB

kinase activity. The PknL activation loop residues Thr173 and Thr175, corresponding to Thr171 and Thr173 in PknB, were also identified as necessary for full PknL activity, and phosphorylation of Thr173 was required for optimal PknL-mediated phosphorylation of Rv2175c (5). Conservation of the autophosphorylation pattern in the activation loop of PknF has been reported, with Thr173 and Thr175 being the only two phosphorylated residues in the activation loop of this kinase (10). Here we addressed whether phosphorylation of the activation loop affects PknF activity by individually substituting Thr173 and Thr175 by Ala to generate the PknF(T173A) and PknF(T175A) mutants, respectively (Tables 1 and 2). In addi-

TABLE 2. Primers used in this study

Primer	Gene	Sequence ^a (5' to 3')
NtermGroEL1	<i>groEL1</i>	ATA TAG CTC <u>ATA</u> TGA GCA AGC TGA TC (NdeI)
CtermGroEL1	<i>groEL1</i>	ATA GGA <u>TCC</u> TCA GTG CGC GTG CCC GTG (BamHI)
NtermGroEL1_T25A	<i>groEL1</i>	ATG GAC AAG CTG GCC GAC GCC GTG CGG GTG ACG CTG GGG
CtermGroEL1_T25A	<i>groEL1</i>	CCC CAG CGT CAC CCG CAC GGC GTC GGC CAG CTT GTC CAT
NtermGroEL1_T54A	<i>groEL1</i>	GTT ACC AAC GAC GGC GTC GCG GTG GCA CGT GAG ATC GAG
CtermGroEL1_T54A	<i>groEL1</i>	CTC GAT CTC ACG TGC CAC CGC GAC GCC GTC GTT GGT AAC
NtermPknF_CD	<i>pknF</i>	TAATAGCT <u>CAT</u> ATG CCG CTC GCG GAA GGT TCG (NdeI)
CtermPknF_CD	<i>pknF</i>	TAT <u>AAG</u> <u>CTT</u> TTA CGG TTG CGA CAC CCG CGT (HindIII)
NtermPknF_CD_T173A	<i>pknF</i>	CCA AGC GGA TTG GCC GCC ACA AAC ATG
NtermPknF_CD_T175A	<i>pknF</i>	CCA AGC GGA TTG ACC GCC GCA AAC ATG ACT GTG GGC ACC
CtermPknF_CD_T173A	<i>pknF</i>	GGT GCC CAC AGT CAT GTT TGC GGC GGT CAA TCC GCT TGG
CtermPknF_CD_T175A	<i>pknF</i>	CCA AGC GGA TTG GCC GCC GCA AAC ATG ACT GTG G
NtermPknF_CD_T173A/T175A	<i>pknF</i>	TA ATA GCT <u>CAT</u> ATG CCA CTG GCC GCT GGG GAG ACA (NdeI)
NtermPknF_CD_smeG	<i>pknF_{Msm}</i>	TAT GGA <u>TCC</u> TCA GGC CGC GTG CCT GGC GAC CGG (BamHI)
CtermPknF_CD_smeG	<i>pknF_{Msm}</i>	TA ATA GCT <u>CAT</u> ATG AGC AAG CAG ATT GAA TTC AAC (NdeI)
NtermGroEL1_smeG	<i>groEL1_{Msm}</i>	TAATAGCT <u>GCT</u> <u>AGC</u> TCA GTG AGC GTG GCC GTG GTG (NheI)
CtermGroEL1_smeG	<i>groEL1_{Msm}</i>	GTC GAC AAG CTC GCC GAC ACC GTC AAG GTC ACG CTC GGC
NtermGroEL1_A25T_smeG	<i>groEL1_{Msm}</i>	GCC GAG CGT GAC CTT GAC GGT GTC GGC GAG CTT GTC GAC
CtermGroEL1_A25T_smeG	<i>groEL1_{Msm}</i>	

^a Restriction sites are underlined and specified in the parentheses after the sequence. Mutagenized codons are shown in bold type.

tion, a double mutant, PknF(T173A/T175A) was also created. Substitution of Thr173 by Ala severely impaired the autokinase activity of PknF (Fig. 2A). In contrast, the Thr175A mutant did not exhibit a strongly reduced signal, although it was weaker than the signal of the wild-type kinase. However, when both residues were mutated, the signal almost completely disappeared. Interestingly, the electrophoretic migration profile showed that PknF(K41M) and PknF(T173A/T175A) migrated faster than PknF(WT), PknF(T173A), or PknF(T175A), although they possess the same molecular mass (Fig. 2A). These different electrophoretic mobility properties are very likely due to their different intrinsic phosphorylation states, as already reported for other kinases (5). Together, these data indicate that double phosphorylation of the Thr173 and Thr175 activation loop residues is necessary for optimal kinase activity, although Thr173 appears to play a more prominent role than Thr175 does. These results support the notion that both phosphothreonines participate in the regulatory control of PknF activity.

Recruitment and phosphorylation of GroEL1 is dependent on the activation loop Thr173 and Thr175. The identification of PknF/GroEL1 as a novel kinase/substrate pair in *M. tuberculosis* prompted us to assess the contribution of the PknF activation loop Thr173 and Thr175 residues in the transphosphorylation reaction between the kinase and its substrate. This was achieved by incubating the *M. tuberculosis* GroEL1 substrate with PknF(K41M), PknF(T173A), PknF(T175A), or PknF(T173A/T175A). As expected, PknF(K41M) was unable to phosphorylate GroEL1, indicating that phosphorylation of Lys41 is a prerequisite to the transphosphorylation reaction (Fig. 2B). More importantly, whereas the T173A mutation profoundly decreased the transphosphorylation reaction, T175A did not significantly alter PknF-dependent phosphorylation of GroEL1. Furthermore, GroEL1 could not be phosphorylated by PknF(T173A/T175A), clearly demonstrating that recruitment and phosphorylation of GroEL1 are dependent on both Thr173 and Thr175. Our results indicate that GroEL1 interacts only with the phosphorylated form of PknF and that this interaction is abolished in the double T173A/T175A mutant, suggesting that the TAT phosphopeptide recognition motif is involved in protein-protein interaction between the two partners. These results are consistent with those reported for PknB in which both Thr171 and Thr173 interact with the GarA substrate, thus indicating that both phosphorylated activation loop residues are necessary for optimal PknB-GarA interaction and maximal kinase activity (34).

GroEL1 is phosphorylated on two threonine residues. We next analyzed the phosphoamino acid content of PknF-phosphorylated GroEL1. The protein (5 μ g) was labeled with [γ - 33 P]ATP in vitro, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), excised, and subjected to acid hydrolysis as reported previously (25). GroEL1 was found to be phosphorylated only on threonine residues (Fig. 3A). While experimentally challenging, mass spectrometry technique was then successfully developed to decipher the phosphorylation sites in a sequence-specific fashion. The *M. tuberculosis* GroEL1 protein contains 34 threonine residues. To identify the phosphorylated site(s), recombinant GroEL1 was incubated with cold ATP in the presence of PknF and subjected to mass spectrometry analysis after tryptic digestion.

ProteinPilot database searching software (version 2.0; Applied Biosystems), using the Paragon method with phosphorylation emphasis, was used to detect and identify the phosphorylated peptides. The sequence coverage of the protein was 88%, and phosphorylation occurred only on two peptides, the peptide from positions 22 to 33 (i.e., LADTVRVTLGPR) and the peptide from positions 42 to 57 (i.e., AFGGPTVTNDGVTVAR) with an 80-Da mass increment from 1,296.73 to 1,376.91 Da, and from 1,560.78 to 1,640.74, respectively (monoisotopic mass). The tandem mass spectrometry (MS-MS) spectra unambiguously confirmed the presence of phosphate groups on Thr25 (Fig. 3B) and Thr54 (Fig. 3C), consistent with the phosphoamino analysis (Fig. 3A).

Definitive identification and localization of Thr25 and Thr54 as the two unique phosphorylation sites were achieved by site-directed mutagenesis to introduce single or double mutations that prevent specific phosphorylation (Thr-to-Ala replacements). All mutants (T25A, T54A, and T25A/T54A) were expressed, purified as a His-tagged protein in *E. coli* BL21(DE3)Star harboring plasmid pETPhos_groEL1_T25A, pETPhos_groEL1_T54A, or pETPhos_groEL1_T25A/T54A, and used in an in vitro kinase assay. The recombinant proteins were individually incubated along with [γ - 33 P]ATP and PknF. Equal amounts of proteins were separated by SDS-PAGE and analyzed by autoradiography (Fig. 4A, top panel). Unexpectedly, whereas a significant decrease of the phosphorylation signal was observed with the T25A mutant, phosphorylation of the T54A mutant was not affected (Fig. 4A, bottom panel). However, phosphorylation of the double mutant was completely abrogated compared to phosphorylation of GroEL1(WT), indicating that GroEL1 is phosphorylated only on Thr25 and Thr54. This was further supported by analysis of an additional round of mass spectrometry on GroEL1(T25A/T54A) pre-treated with ATP and PknF. This failed to identify any additional phosphate group that could eventually have arisen as a compensatory mechanism to the loss of the Thr25 and Thr54 phosphorylation sites (data not shown). Overall, these data indicate that Thr25 and Thr54 are not equivalent with respect to phosphorylation by PknF, as Thr25 appears to be the primary phosphorylation site. In the absence of Thr54, GroEL1 still undergoes phosphorylation on the second site, whereas in the absence of Thr25, the protein has almost completely lost its ability to be phosphorylated.

M. tuberculosis GroEL1 has been demonstrated to be an active stimulator of human monocytes, and this effect was dependent on CD14 (21). Structure/function relationship studies of GroEL1 have shown that the signaling activity of the protein, as well as its capacity to interact with CD14 to induce monocyte cytokine secretion, resides in its equatorial domain (33). Interestingly, both phosphorylated Thr residues are present in helical regions of the equatorial domain of GroEL1, and the localization of the two sites in the domain responsible for GroEL1 activity raises the attractive hypothesis that they may play an important role in regulating GroEL1. Further work will be required to assess whether phosphorylation of this structural domain contributes to the control of GroEL1 activity in vitro and in an in vivo infection model.

GroEL1 from *M. smegmatis* is not phosphorylated by PknF. Using a *groEL1* mutant of *M. smegmatis*, Ojha et al. demonstrated that loss of *M. smegmatis* GroEL1 (GroEL1_{Msm}) func-

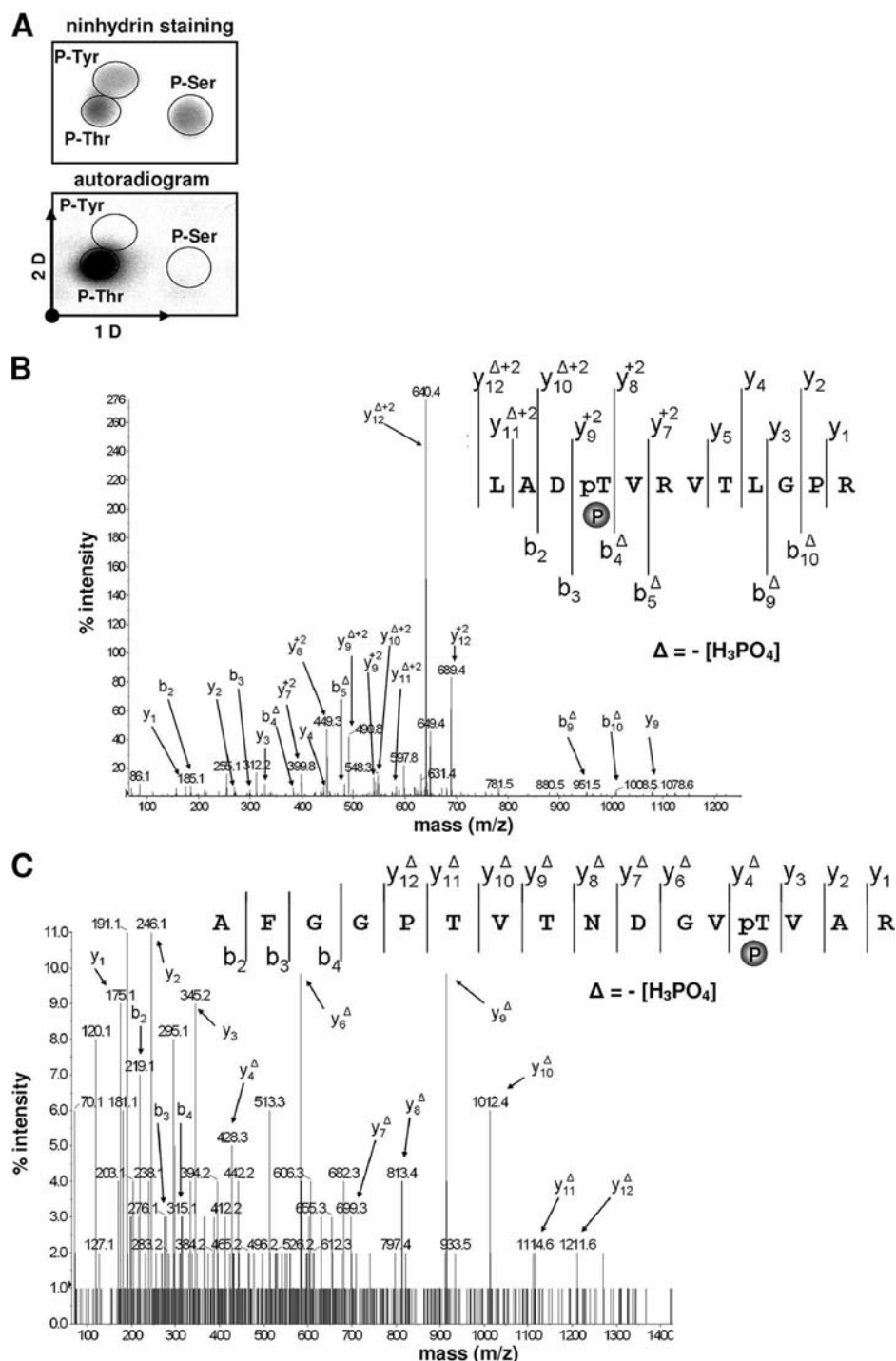


FIG. 3. Identification of the GroEL1 phosphorylation sites. (A) Phosphoamino acid content of the PknF-phosphorylated GroEL1. GroEL1 was phosphorylated *in vitro* in the presence of His-PknF and [γ - ^{33}P]ATP, analyzed by SDS-PAGE, electroblotted onto an Immobilon polyvinylidene difluoride membrane, excised, and hydrolyzed in acid. The phosphoamino acids thus liberated were separated by electrophoresis in the first dimension (1 D) and ascending chromatography in the second dimension (2 D). After migration, radioactive molecules were detected by autoradiography. Authentic phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) were run in parallel as internal standard controls and visualized by ninhydrin staining. (B) MS-MS spectra of the doubly charged ion $[M + 2H]^{2+}$ at m/z 689.36 of the peptide from positions 22 to 33 (monoisotopic mass, 1,376.71 Da) of GroEL1. The unambiguous location of the phosphate group (P) on Thr25 was shown by observation of the “y” C-terminal daughter ion series. Starting from the C-terminal residue, all “y” ions lose phosphoric acid (-98 Da) after the phosphorylated residues. pT, phosphotyrosine. (C) MS-MS spectra of the doubly charged ion $[M + 2H]^{2+}$ at m/z 821.38 of the peptide from positions 42 to 57 (monoisotopic mass, 1,640.74 Da) of GroEL1. The unambiguous location of the phosphate group on Thr54 was shown by observation of the “y” C-terminal daughter ion series. Starting from the C-terminal residue, all “y” ions lose phosphoric acid (-98 Da) after the phosphorylated residues.

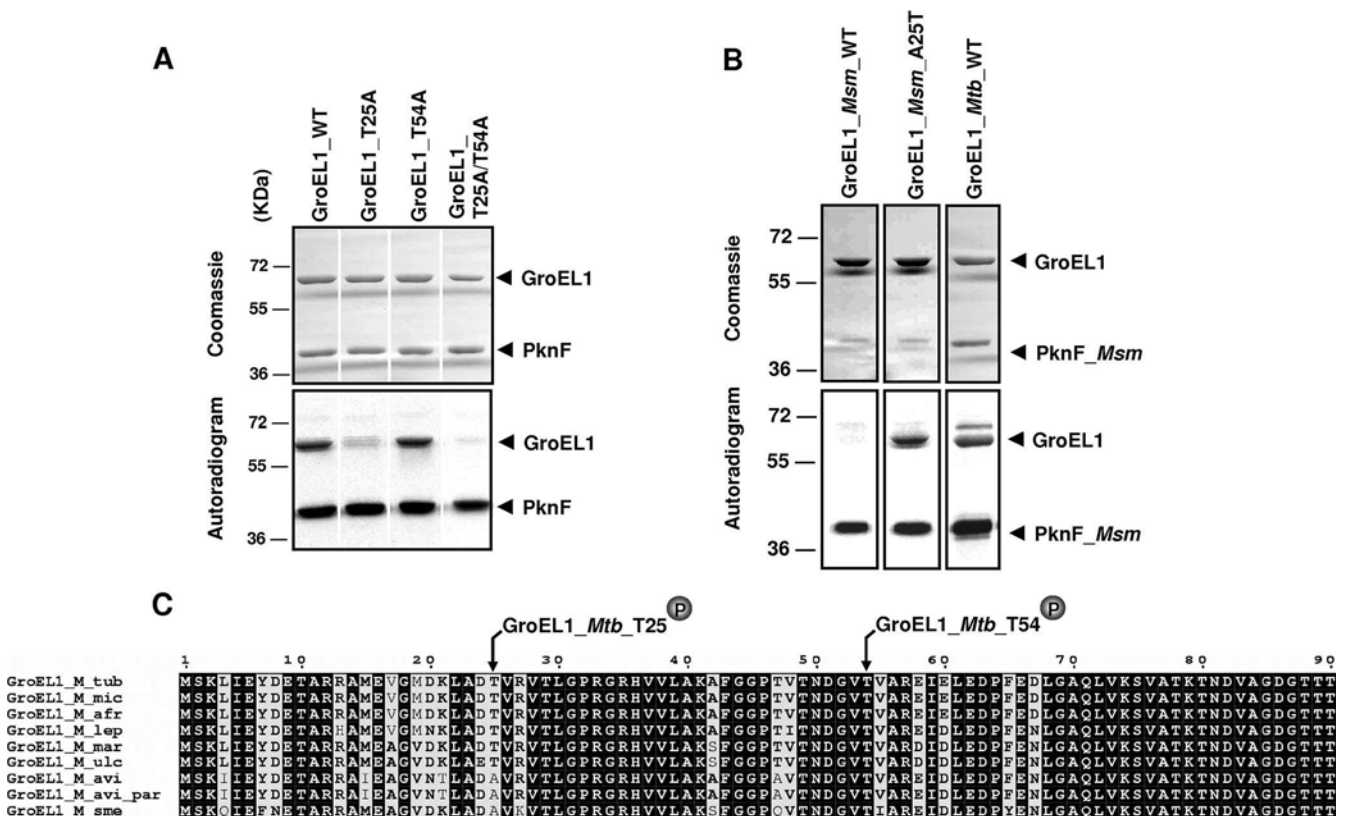


FIG. 4. Differential phosphorylation profiles of GroEL1 proteins from *M. tuberculosis* and *M. smegmatis*. (A) Requirement of both Thr25 and Thr54 for optimal phosphorylation of GroEL1 by PknF. GroEL1(WT) and recombinant GroEL1(T25A), GroEL1(T54A), and GroEL1(T25A T54A) proteins were purified from *E. coli* and used in phosphorylation assays in the presence of purified PknF and [γ - 33 P]ATP. Proteins were then separated by SDS-PAGE and stained with Coomassie blue (top panel), and radioactive bands were revealed by autoradiography (bottom panel). (B) GroEL1 from *M. smegmatis* is not phosphorylated by PknF. Recombinant GroEL1_{Msm}(WT) and GroEL1_{Msm}(A25T) were purified from *E. coli* and used in phosphorylation assays in the presence of purified PknF_{Msm} and [γ - 33 P]ATP. Proteins were then separated by SDS-PAGE and stained with Coomassie blue (top panel), and radioactive bands were revealed by autoradiography (bottom panel). The positions of molecular mass markers (in kilodaltons) are shown to the left of the gels. (C) Sequence alignment of the N-terminal sequences of GroEL1 from various mycobacterial species. The alignment was performed using ClustalW and Esript programs. Residues conserved in all species are shown in white type on black. The phosphorylated sites of GroEL1 are indicated. Abbreviations: M_{tub}, *Mycobacterium tuberculosis*; M_{mic}, *Mycobacterium microti*; M_{afr}, *Mycobacterium africanum*; M_{lep}, *Mycobacterium leprae*; M_{mar}, *Mycobacterium marinum*; M_{ulc}, *Mycobacterium ulcerans*; M_{avi}, *Mycobacterium avium*; M_{avi_par}, *M. paratuberculosis*; M_{sme}, *Mycobacterium smegmatis*.

tion does not affect planktonic growth but specifically interferes with biofilm maturation (28). The behavior of the mutant revealed that mycolic acid synthesis is regulated during biofilm maturation and that GroEL1_{Msm} interacts physically with the condensing enzymes KasA of the fatty acid synthase type II system, a central element of the mycolic acid biosynthetic pathway. Moreover, it has been demonstrated that the condensing activity of KasA is regulated via STPK phosphorylation (23). This prompted us to evaluate and compare the phosphorylation profile of GroEL1_{Msm} incubated with radiolabeled ATP and *M. smegmatis* PknF (PknF_{Msm}). PknF_{Msm} (MSMEG3677) has recently been identified as the closest homologue (64%) to PknF of *M. tuberculosis* (12), and overexpression of PknF_{Msm} in *M. smegmatis* showed reduced growth associated with irregular cell structure and a defect in sliding motility and biofilm formation (12). As shown in Fig. 4B, GroEL1_{Msm} was not phosphorylated by PknF_{Msm} despite a strong autokinase activity for the kinase, whereas *M. tuberculosis* GroEL1 (GroEL1_{Mtb}) was phosphorylated by PknF_{Msm}. This result was rather unexpected, given the 81% identity shared by

GroEL1_{Msm} and GroEL1_{Mtb}. Comparison of the primary sequences of GroEL1_{Msm} and GroEL1_{Mtb} revealed that Thr54 was conserved in both proteins, but Thr25 was substituted by Ala in GroEL1_{Msm} (Fig. 4C). We next examined whether the absence of a threonine at position 25 could explain the lack of phosphorylation of GroEL1_{Msm} by PknF_{Msm}. Site-directed mutagenesis was used to replace Ala25 by Thr25, thus generating GroEL1_{Msm}(A25T). Importantly, the A25T replacement restored the capacity of GroEL1_{Msm} to be phosphorylated by PknF_{Msm} (Fig. 4B), thus connecting the lack of phosphorylation of GroEL1_{Msm} to the absence of a threonine residue at position 25.

Together, these results suggest the presence of various degrees of interaction between GroEL1 proteins and mycobacterial kinases. This is further supported by the observation that GroEL1_{Msm} could be phosphorylated to some extent by several *M. tuberculosis* STPKs (including PknA, PknB, PknE, and PknF) in vitro, thus pointing out a possible cross talk between mycobacterial species (data not shown).

Our study indicates that GroEL1 is differentially phosphor-

ylated in various mycobacterial species. Whether these different GroEL1 isoforms affect/regulate the function(s) of GroEL1 remains to be investigated, but it is anticipated that GroEL1 expresses different activities that depend directly on its phosphorylation profile, and hence on the species in which the GroEL1 is synthesized. Interestingly, the Thr25 residue was found to be conserved in all pathogenic species analyzed, including all members of the *Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, *Mycobacterium marinum*, and *Mycobacterium ulcerans* (Fig. 4C). One could therefore hypothesize that this particular residue is somehow critical for GroEL1 activity in pathogenic species.

In conclusion, although mycobacterial GroEL1 proteins have been extensively studied, no data were available with respect to their potential posttranslational modifications. We report here, for the first time, phosphorylation of the *M. tuberculosis* GroEL1 chaperone and identified Thr25 as a critical phosphoacceptor. The results of the present study suggest that environmental signals could trigger GroEL1 phosphorylation and presumably influence/regulate the biological activity of the protein. Whether polymorphism at position 25 contributes to the different functions associated to GroEL1 in pathogenic versus nonpathogenic species is an attractive hypothesis that remains to be addressed. The generation of an isogenic strain of *M. tuberculosis* possessing a *groEL1(T25A)* allele would be necessary in order to examine the putative link between Thr25 phosphorylation and the capacity to induce an inflammatory response in infected animals. Conversely, whether the phosphorylation profile of GroEL1(A25T) alters the interaction with KasA in *M. smegmatis* and subsequently influences the production of mature biofilms would also be of interest. In contrast to *groEL1*, *groEL2* is essential for mycobacterial growth (28). We have thus investigated whether GroEL2 also represents a substrate for *M. tuberculosis* kinases. The results of our preliminary studies suggest that GroEL2 can be phosphorylated in vitro (data not shown). Work is currently under progress to identify the phosphorylated site(s) in GroEL2 and to determine how phosphorylation may affect the GroEL2 functions.

We thank M. Becchi, I. Zanella-Cléon, and A. Cornut (IBCP, Lyon, France) for their excellent expertise and technical assistance in mass spectrometry analysis.

This work was supported by grants from the Region Rhone-Alpes (M.J.C.), the CNRS, the University of Lyon (France), and the National Research Agency (ANR-06-MIME-027-01 to V.M. and L.K.).

REFERENCES

- Av-Gay, Y., and M. Everett. 2000. The eukaryotic-like Ser/Thr protein kinases of *Mycobacterium tuberculosis*. *Trends Microbiol.* **8**:238–244.
- Bhatt, A., V. Molle, G. S. Besra, W. R. Jacobs, Jr., and L. Kremer. 2007. The *Mycobacterium tuberculosis* FAS-II condensing enzymes: their role in mycolic acid biosynthesis, acid-fastness, pathogenesis and in future drug development. *Mol. Microbiol.* **64**:1442–1454.
- Boitel, B., M. Ortiz-Lombardia, R. Duran, F. Pompeo, S. T. Cole, C. Cervenansky, and P. M. Alzari. 2003. PknB kinase activity is regulated by phosphorylation in two Thr residues and dephosphorylation by PstP, the cognate phospho-Ser/Thr phosphatase, in *Mycobacterium tuberculosis*. *Mol. Microbiol.* **49**:1493–1508.
- Canova, M. J., L. Kremer, and V. Molle. 2008. pETPhos: a customized expression vector designed for further characterization of Ser/Thr/Tyr protein kinases and their substrates. *Plasmid* **60**:149–153.
- Canova, M. J., R. Veyron-Churlet, I. Zanella-Cléon, M. Cohen-Gonsaud, A. J. Cozzone, M. Becchi, L. Kremer, and V. Molle. 2008. The *Mycobacterium tuberculosis* serine/threonine kinase PknL phosphorylates Rv2175c: mass spectrometric profiling of the activation loop phosphorylation sites and their role in the recruitment of Rv2175c. *Proteomics* **8**:521–533.
- Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**:537–544.
- Curry, J. M., R. Whalan, D. M. Hunt, K. Gohil, M. Strom, L. Rickman, M. J. Colston, S. J. Smerdon, and R. S. Buxton. 2005. An ABC transporter containing a forkhead-associated domain interacts with a serine-threonine protein kinase and is required for growth of *Mycobacterium tuberculosis* in mice. *Infect. Immun.* **73**:4471–4477.
- Deol, P., R. Vohra, A. K. Saini, A. Singh, H. Chandra, P. Chopra, T. K. Das, A. K. Tyagi, and Y. Singh. 2005. Role of *Mycobacterium tuberculosis* Ser/Thr kinase PknF: implications in glucose transport and cell division. *J. Bacteriol.* **187**:3415–3420.
- Dosanjh, N. S., M. Rawat, J. H. Chung, and Y. Av-Gay. 2005. Thiol specific oxidative stress response in mycobacteria. *FEMS Microbiol. Lett.* **249**:87–94.
- Duran, R., A. Villarino, M. Bellinzoni, A. Wehenkel, P. Fernandez, B. Boitel, S. T. Cole, P. M. Alzari, and C. Cervenansky. 2005. Conserved autophosphorylation pattern in activation loops and juxtamembrane regions of *Mycobacterium tuberculosis* Ser/Thr protein kinases. *Biochem. Biophys. Res. Commun.* **333**:858–867.
- Fayet, O., T. Ziegelhoffer, and C. Georgopoulos. 1989. The *groES* and *groEL* heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *J. Bacteriol.* **171**:1379–1385.
- Gopaldaswamy, R., S. Narayanan, W. R. Jacobs, Jr., and Y. Av-Gay. 2008. *Mycobacterium smegmatis* biofilm formation and sliding motility are affected by the serine/threonine protein kinase PknF. *FEMS Microbiol. Lett.* **278**:121–127.
- Grundner, C., L. M. Gay, and T. Alber. 2005. *Mycobacterium tuberculosis* serine/threonine kinases PknB, PknD, PknE, and PknF phosphorylate multiple FHA domains. *Protein Sci.* **14**:1918–1921.
- Hakansson, S., E. E. Galyov, R. Rosqvist, and H. Wolf-Watz. 1996. The *Yersinia* YpkA Ser/Thr kinase is translocated and subsequently targeted to the inner surface of the HeLa cell plasma membrane. *Mol. Microbiol.* **20**:593–603.
- Hu, Y., B. Henderson, P. A. Lund, P. Tormay, M. T. Ahmed, S. S. Gurucha, G. S. Besra, and A. R. Coates. 2008. A *Mycobacterium tuberculosis* mutant lacking the *groEL* homologue *cpn60.1* is viable but fails to induce an inflammatory response in animal models of infection. *Infect. Immun.* **76**:1535–1546.
- Huse, M., and J. Kuriyan. 2002. The conformational plasticity of protein kinases. *Cell* **109**:275–282.
- Johnson, L. N., M. E. Noble, and D. J. Owen. 1996. Active and inactive protein kinases: structural basis for regulation. *Cell* **85**:149–158.
- Kerner, M. J., D. J. Naylor, Y. Ishihama, T. Maier, H. C. Chang, A. P. Stines, C. Georgopoulos, D. Frishman, M. Hayer-Hartl, M. Mann, and F. U. Hartl. 2005. Proteome-wide analysis of chaperonin-dependent protein folding in *Escherichia coli*. *Cell* **122**:209–220.
- Kim, A. I., P. Ghosh, M. A. Aaron, L. A. Bibb, S. Jain, and G. F. Hatfull. 2003. Mycobacteriophage Bxb1 integrates into the *Mycobacterium smegmatis* *groEL1* gene. *Mol. Microbiol.* **50**:463–473.
- Kong, T. H., A. R. Coates, P. D. Butcher, C. J. Hickman, and T. M. Shinnick. 1993. *Mycobacterium tuberculosis* expresses two chaperonin-60 homologs. *Proc. Natl. Acad. Sci. USA* **90**:2608–2612.
- Lewthwaite, J. C., A. R. Coates, P. Tormay, M. Singh, P. Mascagni, S. Poole, M. Roberts, L. Sharp, and B. Henderson. 2001. *Mycobacterium tuberculosis* chaperonin 60.1 is a more potent cytokine stimulator than chaperonin 60.2 (Hsp 65) and contains a CD14-binding domain. *Infect. Immun.* **69**:7349–7355.
- Lund, P. A. 2001. Microbial molecular chaperones. *Adv. Microb. Physiol.* **44**:93–140.
- Molle, V., A. K. Brown, G. S. Besra, A. J. Cozzone, and L. Kremer. 2006. The condensing activities of the *Mycobacterium tuberculosis* type II fatty acid synthase are differentially regulated by phosphorylation. *J. Biol. Chem.* **281**:30094–30103.
- Molle, V., C. Girard-Blanc, L. Kremer, P. Doublet, A. J. Cozzone, and J. F. Prost. 2003. Protein PknE, a novel transmembrane eukaryotic-like serine/threonine kinase from *Mycobacterium tuberculosis*. *Biochem. Biophys. Res. Commun.* **308**:820–825.
- Molle, V., L. Kremer, C. Girard-Blanc, G. S. Besra, A. J. Cozzone, and J. F. Prost. 2003. An FHA phosphoprotein recognition domain mediates protein EmBR phosphorylation by PknH, a Ser/Thr protein kinase from *Mycobacterium tuberculosis*. *Biochemistry* **42**:15300–15309.
- Molle, V., D. Soulat, J. M. Jault, C. Grangeasse, A. J. Cozzone, and J. F. Prost. 2004. Two FHA domains on an ABC transporter, Rv1747, mediate its

- phosphorylation by PknF, a Ser/Thr protein kinase from *Mycobacterium tuberculosis*. FEMS Microbiol. Lett. **234**:215–223.
27. **Monahan, I. M., J. Betts, D. K. Banerjee, and P. D. Butcher.** 2001. Differential expression of mycobacterial proteins following phagocytosis by macrophages. Microbiology **147**:459–471.
 28. **Ojha, A., M. Anand, A. Bhatt, L. Kremer, W. R. Jacobs, Jr., and G. F. Hatfull.** 2005. GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria. Cell **123**:861–873.
 29. **Orme, I. M., A. D. Roberts, J. P. Griffin, and J. S. Abrams.** 1993. Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. J. Immunol. **151**:518–525.
 30. **Qamra, R., S. C. Mande, A. R. Coates, and B. Henderson.** 2005. The unusual chaperonins of *Mycobacterium tuberculosis*. Tuberculosis (Edinburgh) **85**: 385–394.
 31. **Qamra, R., V. Srinivas, and S. C. Mande.** 2004. *Mycobacterium tuberculosis* GroEL homologues unusually exist as lower oligomers and retain the ability to suppress aggregation of substrate proteins. J. Mol. Biol. **342**:605–617.
 32. **Stewart, G. R., L. Wernisch, R. Stabler, J. A. Mangan, J. Hinds, K. G. Laing, D. B. Young, and P. D. Butcher.** 2002. Dissection of the heat-shock response in *Mycobacterium tuberculosis* using mutants and microarrays. Microbiology **148**:3129–3138.
 33. **Tormay, P., A. R. Coates, and B. Henderson.** 2005. The intercellular signaling activity of the *Mycobacterium tuberculosis* chaperonin 60.1 protein resides in the equatorial domain. J. Biol. Chem. **280**:14272–14277.
 34. **Villarino, A., R. Duran, A. Wehenkel, P. Fernandez, P. England, P. Brodin, S. T. Cole, U. Zimny-Arndt, P. R. Jungblut, C. Cervenansky, and P. M. Alzari.** 2005. Proteomic identification of *M. tuberculosis* protein kinase substrates: PknB recruits GarA, a FHA domain-containing protein, through activation loop-mediated interactions. J. Mol. Biol. **350**:953–963.
 35. **Wang, J., C. Li, H. Yang, A. Mushegian, and S. Jin.** 1998. A novel serine/threonine protein kinase homologue of *Pseudomonas aeruginosa* is specifically inducible within the host infection site and is required for full virulence in neutropenic mice. J. Bacteriol. **180**:6764–6768.
 36. **Wehenkel, A., M. Bellinzoni, M. Grana, R. Duran, A. Villarino, P. Fernandez, G. Andre-Leroux, P. England, H. Takiff, C. Cervenansky, S. T. Cole, and P. M. Alzari.** 2008. Mycobacterial Ser/Thr protein kinases and phosphatases: physiological roles and therapeutic potential. Biochim. Biophys. Acta **1784**: 193–202.

Article IV

The *Mycobacterium tuberculosis* GroEL1 chaperone is a substrate of Ser/Thr Protein Kinases

Canova MJ, Kremer L, and Molle V

Journal of Bacteriology 2009, 191: 2876-2883

Présentation de l'article IV :

Au sein de la cellule, les protéines chaperonnes GroEL sont des acteurs clés du contrôle du repliement des protéines, ainsi que de l'adressage post-traductionnel de ces protéines. Bien que la plupart des bactéries ne possèdent qu'un seul gène *groEL* dans leur génome, *M. tuberculosis* a la particularité de synthétiser deux chaperonnes nommées GroEL1 et GroEL2. Ces deux protéines sont impliquées dans différents mécanismes cellulaires comme la réponse au choc thermique ou au stress oxydant, ainsi que dans la réponse immunitaire lors de l'infection dans les macrophages.

Dans cette étude, nous avons montré que GroEL1 était phosphorylée par PknF, une des 11 STPKs de *M. tuberculosis*. De même que pour le couple PknL/Rv2175c, des analyses par spectrométrie de masse et mutagenèse dirigée ont permis de mettre en évidence le caractère essentiel des résidus Thr173 et Thr175 de la boucle d'activation de PknF sur son activité d'autophosphorylation. Nous avons également montré que le recrutement et la phosphorylation de GroEL1 étaient étroitement liés à la présence du résidu Thr173 de la boucle d'activation de PknF. De plus, les résidus Thr25 et Thr54 ont été identifiés par spectrométrie de masse comme étant les sites de phosphorylation de GroEL1, et des expériences de mutagenèse dirigée nous ont permis de confirmer cette identification.

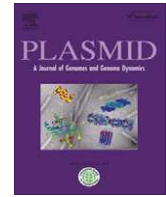
De manière intéressante, le résidu Thr25 est conservé chez les chaperonnes d'autres espèces de mycobactéries comme *M. leprae* et *M. ulcerans*, mais ne l'est pas dans la séquence du GroEL1 de *M. smegmatis*, souche non pathogène (Thr remplacé par Ala). De ce fait, il était intéressant d'étudier ce couple PknF/GroEL1 chez *M. smegmatis*, et cette étude a mis en évidence la non-phosphorylation de GroEL1 dans cette souche.

Par conséquent, cette étude a permis de montrer que GroEL1 était différemment phosphorylée dans différentes espèces de mycobactéries, et soulève la possibilité d'un rôle original de régulation via phosphorylation spécifique aux souches pathogènes.



Contents lists available at ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Short Communication

pETPhos: A customized expression vector designed for further characterization of Ser/Thr/Tyr protein kinases and their substrates

Marc J. Canova^a, Laurent Kremer^{b,c}, Virginie Molle^{a,*}

^aInstitut de Biologie et Chimie des Protéines (IBCP UMR 5086), CNRS, Université Lyon1, IFR128 BioSciences, Lyon-Gerland, 7 passage du Vercors, 69367 Lyon Cedex 07, France

^bLaboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, Université de Montpellier II et I, CNRS, UMR 5235, case 107, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France

^cINSERM, DIMNP, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France

ARTICLE INFO

Article history:

Received 22 April 2008

Revised 16 May 2008

Available online 1 July 2008

Communicated by Julian Rood

Keywords:

Ser/Thr/Tyr protein kinase
Phosphorylation
TEV protease-cleavable tags
Mycobacterium tuberculosis
PknL

ABSTRACT

Bacterial genomics revealed the widespread distribution of serine/threonine protein kinases (STPKs), which regulate various cellular processes. However, understanding the role of phosphorylation in prokaryotes has been hampered by the paucity of endogenous substrates identified and the restricted number of tools allowing identification and characterization of the phosphoresidues. Herein, we describe an improved vector, pETPhos, to express proteins harboring a N-terminal His-tag fusion, which can be efficiently removed using the TEV protease. One major advantage of pETPhos relies on the lack of Ser and Thr residues in the fusion tag, representing potential non-specific phosphorylation sites. The usefulness of pETPhos is illustrated by a comparative analysis in which the *Mycobacterium tuberculosis* protein Rv2175c, a substrate of the STPK PknL, is expressed either in a pET28 derivative or in pETPhos. Following *in vitro* phosphorylation with PknL, phosphoaminoacid analysis revealed the presence of phosphorylated Ser and Thr in Rv2175c expressed in the pET28 derivative. However, when expressed in pETPhos, only Thr were phosphorylated. These findings indicate that STPKs can phosphorylate Ser-containing His-tag fusions, thus conducting to misleading results. We demonstrate that pETPhos represents a valuable tool for characterization of the phosphoacceptors in bacterial STPKs, and presumably also in Tyr protein kinases, as well as in their substrates.

© 2008 Elsevier Inc. All rights reserved.

Reversible protein phosphorylation is a key mechanism by which environmental signals are transmitted to cause changes in protein expression or activity in both eukaryotes and prokaryotes, and therefore is an important mechanism by which extracellular signals are translated into cellular responses. Genes encoding functional serine/threonine protein kinases (STPKs) are ubiquitous in prokaryotic genomes, and signaling through Ser/Thr phosphorylation has emerged as a critical regulatory mechanism in various microorganisms.

In bacteria, the presence of several STPKs suggests a central role of protein phosphorylation in regulating vari-

ous biological functions, ranging from environmental adaptive responses to bacterial pathogenicity. For instance, *Mycobacterium tuberculosis* contains 11 different STPKs (Av-Gay and Everett, 2000; Cole et al., 1998) and most are being investigated for their physiological roles and potential application for future drug development to combat tuberculosis (Wehenkel et al., 2008). However, understanding prokaryotic kinase biology has been seriously hampered by the failure to identify relevant kinase substrates and to identify the phosphorylation site(s) in these substrates. We and others have previously reported the identification and characterization of several *M. tuberculosis* STPK substrates, which belong to various biosynthetic pathways (Canova et al., 2008; Greenstein et al., 2007; Molle et al., 2003, 2006, 2008; Sharma et al., 2006; Villari-

* Corresponding author. Fax: +33 4 72 72 26 41.
E-mail address: vmolle@ibcp.fr (V. Molle).

no et al., 2005; Zheng et al., 2007). The mechanisms by which phosphorylation influences and/or controls the activity of the STPKs kinases and their endogenous substrates in bacteria remain largely unknown, essentially due to the difficulty of identifying the phosphorylation site(s). One strategy to uncover the phosphoacceptors in a protein relies on the purification of the corresponding recombinant His-tagged protein, which can be subsequently phosphorylated *in vitro* in the presence of a purified kinase and ATP. Mass spectrometry can then be used to identify the phosphoresidues, and definitive identification and localization of the phosphorylated sites can be achieved by site-directed mutagenesis. However, this method presents important limitations, inherent to the presence of Ser and Thr residues in most of the His-tag fusions used to overexpress and purify recombinant proteins. These sites can represent non-specific phosphate acceptors in *in vitro* phosphorylation assays (Boitel et al., 2003; Canova et al., 2008). Thus, non-specific phosphorylation can lead to false-positive results in proteins, which are not real STPK substrates, and can also hamper subsequent identification of the specific phosphoacceptors. As previously shown, mass spectrometry analysis of the *M. tuberculosis* PknB kinase expressed in pET28 unambiguously detected the presence of a phosphorylated His-tag peptide (Boitel et al., 2003). Therefore, improved vectors are required to encounter these problems and to allow a more rapid and satisfactory identification of the phosphorylation sites in both prokaryotic kinases and kinase substrates.

Here, we report the construction of an improved pET expression vector, named pETPhos, that (i) directs synthesis of recombinant proteins possessing a N-terminal His-tag fusion, (ii) contains a very efficient TEV protease cleavage site, and more importantly (iii) is devoid of putative phosphoacceptors in the His-tag fusion. We provide evidence that pETPhos is particularly suited for the overexpression and purification of recombinant proteins for further identification of the phosphorylation sites.

Because the presence of a His-tag represents a possible source of error in the interpretation of the data on protein phosphorylation, we have designed and constructed an improved vector, pETPhos, to express proteins harboring a N-terminal His-tag fusion, which can be efficiently removed using the tobacco etch virus (TEV) protease. To construct the pETPhos vector, site-directed mutagenesis was carried out based on PCR amplification with the QuickChange site-directed mutagenesis kit (Stratagene). The strategy consisted in creating substitutions in the N-terminal His-tag sequence of the pETtev plasmid, a variant of pET15b (Novagen) that includes the replacement of the thrombin site coding sequence with a tobacco etch virus (TEV) protease site (Cohen-Gonsaud et al., 2004). The four Ser residues at positions 3, 4, 11 and 12 with respect to the Met residue at position one in the N-terminal tag of the pETtev vector were mutagenized for Gly residues. The pETPhos plasmid was constructed using pETtev as template with the following primers: #566, 5'-ATG GGC GGC CAC CAT CAT CAT CAT CAT CAC GGC GGC GGC-3' and #567, 5'-GCC GCC GCC GTG ATG ATG ATG ATG ATG GTG GCC GCC CAT-3'. Fig. 1 represents the restriction map of the vector as well as the nucleotidic sequence of the pETPhos cloning/expression

region, along with the restriction endonuclease sites. One major advantage of this vector relies on the lack of Ser and Thr residues in the fusion tag, representing potential non-specific phosphorylation sites.

The usefulness of pETPhos is illustrated below by a comparative analysis, in which the *M. tuberculosis* protein Rv2175c, a substrate of the STPK PknL (Canova et al., 2008), is expressed either in a pET28 derivative or in pETPhos. Rv2175c possesses weak similarity to transcriptional regulatory proteins harboring a DNA-binding domain (residues 32–52) constituted of a helix-turn-helix (HTH) motif. Therefore, the Rv2175c/PknL pair has been proposed to participate in regulating the neighboring genes in the division and cell wall biosynthesis (*dcw*) cluster (Narayan et al., 2007). In this study, Rv2175c was chosen as a kinase substrate model protein in order to investigate the deleterious effect of the N-terminal His-tag fusion during analysis and characterization of the phosphorylation sites. To this aim, recombinant Rv2175c proteins were expressed in *Escherichia coli* as two variants possessing different N-terminal fusion tags. The *Rv2175c* gene was amplified by PCR using *M. tuberculosis* H37Rv genomic DNA as a template and the following primers: #403, 5'-TAT ATA TCG TT CAT ATG CCT GGC CGC GCA CCA GGC TCT-3' (containing a NdeI restriction site underlined) and, #404, 5'-TAT GGA TCC TCA ATA CGC CAT AGC CTG GGC CCG-3' (containing a BamHI restriction site underlined). This 441-bp amplified product was digested by NdeI and BamHI, and ligated into pET28Amp and pETPhos, respectively. The pET28Amp vector is a pET28a (Novagen) derivative, that includes the replacement of the kanamycin resistance cassette with a ampicillin cassette (Molle et al., 2006). The *pknL* PCR fragment encoding the cytoplasmic domain (CD) corresponding to the kinase domain and the juxtamembrane linker of PknL (residues 1–369 out of 399) was also amplified by PCR using *M. tuberculosis* H37Rv genomic DNA as a template and the following primers: #196, 5'-TAT GGA TCC GTG GTC GAA GCT GGC ACG-3' (containing a BamHI restriction site underlined) and, #197, 5'-TAT AAG CTT TTA TCG ACG GGC GTG CTG TCG-3' (containing a HindIII restriction site underlined). This 1107-bp amplified product was digested by BamHI and HindIII, and ligated into pET28Amp, thus generating pET28Amp-*pknL*_{CD}. Recombinant *E. coli* BL21(DE3) Star (Stratagene) strains harboring the kinase-expressing construct pET28Amp-*pknL*_{CD}, the Rv2175c-expressing constructs pET28Amp-*Rv2175c* or pETPhos-*Rv2175c*, were used to inoculate 200 ml of LB medium supplemented with 100 µg/ml ampicillin and incubated at 25 °C with shaking. When A_{600} reached 0.5, IPTG was added at a final concentration of 0.5 mM, and growth was continued for an additional 3 h at room temperature with shaking. Purifications of the His-tagged proteins were performed as reported previously (Molle et al., 2006). When required, the protein was treated with TEV protease according to the manufacturer's instructions (Invitrogen).

As shown in Fig. 2A, Rv2175c_pET28Amp contains an N-terminal His-tag bearing five Ser residues and a thrombin cleavage site, whereas Rv2175c_pETPhos possesses an N-terminal His-tag lacking any Ser or Thr residues, and which can subsequently be removed by TEV protease cleavage. TEV protease has become one of the enzymes of

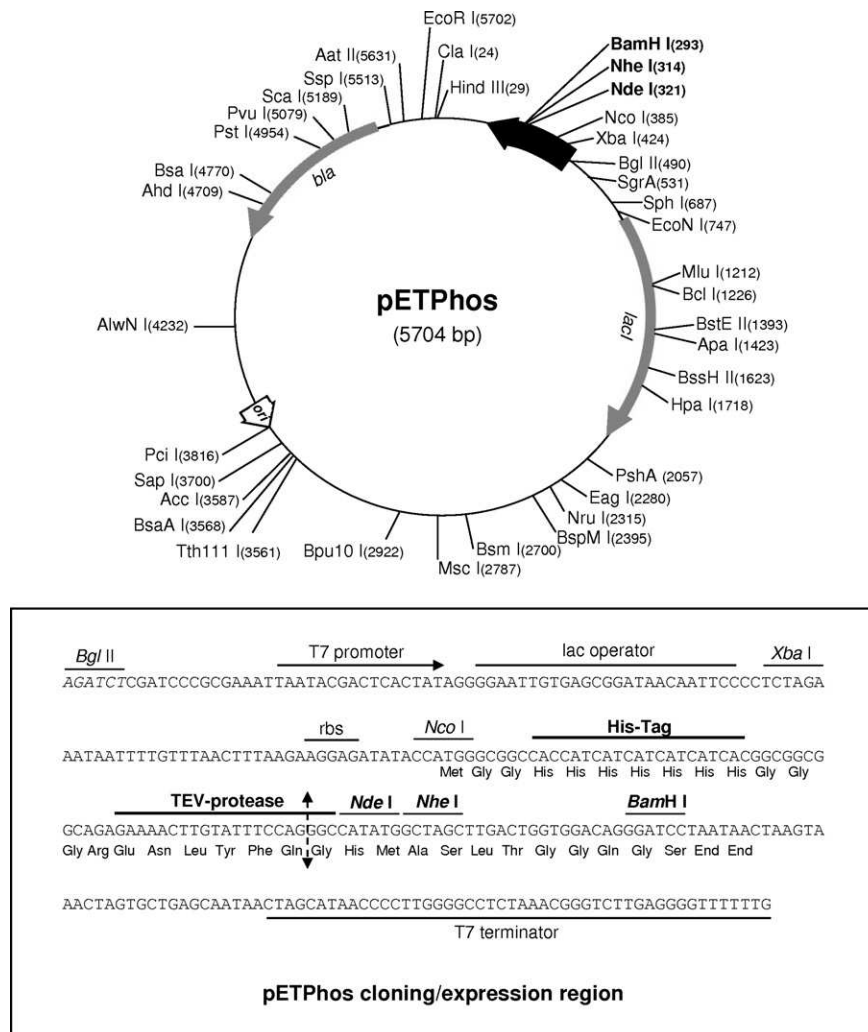


Fig. 1. Map of the cloning vector pETPhos. The pETPhos vector carries an N-terminal His-tag sequence followed by a TEV protease cleavage site and multiple cloning sites. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so that the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below.

choice for tag removal in fusion proteins due to its high degree of specificity, its resistance to many protease inhibitors used during the purification process, and the ease of separation of the TEV protease from the protein of interest (Parks et al., 1994). Additionally, improvement in the ability to purify large amounts of TEV protease can make it a relatively inexpensive choice to other commercially available options (Blommel and Fox, 2007; van den Berg et al., 2006). After purification on Ni-NTA agarose beads, Rv2175c_pET28Amp and Rv2175c_pETPhos were treated with thrombin and TEV protease, respectively. Tagged and untagged Rv2175c variants were next analyzed by SDS-PAGE, allowing separation of the different protein forms and to evaluate the efficiency of proteolytic cleavage. TEV cleavage performed either directly on the soluble protein or “on-column” whilst the protein is still bound to the matrix proved to be very efficient (data not shown).

The different Rv2175c variants were then assayed in the presence of [γ - ^{33}P]ATP and purified recombinant PknL_{CD},

consisting of the cytoplasmic domain comprising the kinase domain and the juxtamembrane linker of PknL. *In vitro* phosphorylation of Rv2175c_pET28Amp (3 μg) or Rv2175c_pETPhos (3 μg) by PknL_{CD} (0.5 μg) was carried out for 30 min at 37 °C in a reaction mixture (20 μl) containing buffer P (25 mM Tris-HCl, pH 7.0; 1 mM DTT; 5 mM MgCl₂; 1 mM EDTA) and 200 $\mu\text{Ci/ml}$ [γ - ^{33}P]ATP (PerkinElmer, 3000 Ci/mmol). The reaction was stopped by adding an equal volume of 2 \times sample buffer and boiling the mixture for 5 min. After electrophoresis, gels were soaked in 20% TCA for 10 min at 90 °C and dried. Radioactive proteins were visualized by autoradiography using direct exposure to films. As shown in Fig. 2B (lane 1), PknL_{CD} could phosphorylate Rv2175c_pET28Amp, in agreement with our previous observations (Canova et al., 2008). No phosphorylation of Rv2175c was observed in the absence of PknL_{CD} (data not shown, Canova et al., 2008). Thrombin treatment led to a partially processed Rv2175c protein, as shown by the presence of a doublet, corresponding to the

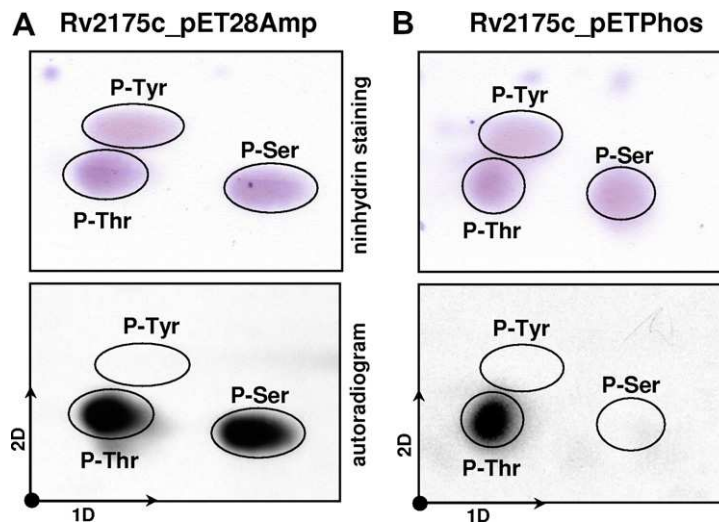


Fig. 3. Phosphoamino acid content of non-cleaved Rv2175c_pET28Amp and Rv2175c_pETPhos proteins. Proteins labeled with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ were analyzed by SDS-PAGE, electroblotted onto an Immobilon PVDF membrane, excised and hydrolyzed in acid. The phosphoamino acids thus liberated were separated by electrophoresis in the first dimension (1D) and ascending chromatography in the second dimension (2D). After migration, radioactive molecules were detected by autoradiography. Authentic phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) were run in parallel as internal standard controls, and visualized by ninhydrin staining. (A) Phosphoamino acid content of Rv2175c_pET28Amp phosphorylated by PknLCD. (B) Phosphoamino acid content of Rv2175c_pETPhos phosphorylated by PknLCD.

overcome these problems, by combining a Ser/Thr-free N-terminal His-tag, along with a TEV protease cleavage site to allow efficient removal of the tag if required. We therefore propose that pETPhos could be a valuable tool to facilitate the identification of the phosphorylation sites, not only in STPKs, but presumably also in Tyr protein kinases, as well as in their substrates without inconvenience of non-specific phosphorylation.

Acknowledgments

The authors wish to acknowledge Dr. M. Cohen-Gonsaud for the generous gift of the pETTeV vector. This work was supported by Grants from the Region Rhone-Alpes (M.C.), the CNRS, the University of Lyon (France), and the National Research Agency (ANR-06-MIME-027-01 to V.M. and L.K.).

References

Av-Gay, Y., Everett, M., 2000. The eukaryotic-like Ser/Thr protein kinases of *Mycobacterium tuberculosis*. *Trends Microbiol.* 8, 238–244.
 Blommel, P.G., Fox, B.G., 2007. A combined approach to improving large-scale production of tobacco etch virus protease. *Protein Expr. Purif.* 55, 53–68.
 Boitel, B. et al, 2003. PknB kinase activity is regulated by phosphorylation in two Thr residues and dephosphorylation by PstP, the cognate phospho-Ser/Thr phosphatase, in *Mycobacterium tuberculosis*. *Mol. Microbiol.* 49, 1493–1508.
 Canova, M.J. et al, 2008. The *Mycobacterium tuberculosis* serine/threonine kinase PknL phosphorylates Rv2175c: mass spectrometric profiling of the activation loop phosphorylation sites and their role in the recruitment of Rv2175c. *Proteomics* 8, 521–533.

Cohen-Gonsaud, M. et al, 2004. (1)H, (15)N, and (13)C chemical shift assignments of the resuscitation promoting factor domain of Rv1009 from *Mycobacterium tuberculosis*. *J. Biomol. NMR* 30, 373–374.
 Cole, S.T. et al, 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393, 537–544.
 Greenstein, A.E. et al, 2007. *M. tuberculosis* Ser/Thr protein kinase D phosphorylates an anti-anti-sigma factor homolog. *PLoS Pathog.* 3, e49.
 Molle, V. et al, 2003. An FHA phosphoprotein recognition domain mediates protein EmbR phosphorylation by PknH, a Ser/Thr protein kinase from *Mycobacterium tuberculosis*. *Biochemistry* 42, 15300–15309.
 Molle, V. et al, 2006. The condensing activities of the *Mycobacterium tuberculosis* type II fatty acid synthase are differentially regulated by phosphorylation. *J. Biol. Chem.* 281, 30094–30103.
 Molle, V. et al, 2008. EmbR2, a structural homologue of EmbR, inhibits the *Mycobacterium tuberculosis* kinase/substrate pair PknH/EmbR. *Biochem. J.* 410, 309–317.
 Narayan, A. et al, 2007. Serine threonine protein kinases of mycobacterial genus: phylogeny to function. *Physiol. Genomics* 29, 66–75.
 Parks, T.D. et al, 1994. Release of proteins and peptides from fusion proteins using a recombinant plant virus proteinase. *Anal. Biochem.* 216, 413–417.
 Sharma, K. et al, 2006. Transcriptional control of the mycobacterial embCAB operon by PknH through a regulatory protein, EmbR, *in vivo*. *J. Bacteriol.* 188, 2936–2944.
 van den Berg, S. et al, 2006. Improved solubility of TEV protease by directed evolution. *J. Biotechnol.* 121, 291–298.
 Villarino, A. et al, 2005. Proteomic identification of *M. tuberculosis* protein kinase substrates: PknB recruits GarA, a FHA domain-containing protein, through activation loop-mediated interactions. *J. Mol. Biol.* 350, 953–963.
 Wehenkel, A. et al, 2008. Mycobacterial Ser/Thr protein kinases and phosphatases: physiological roles and therapeutic potential. *Biochim. Biophys. Acta* 1784, 193–202.
 Zheng, X. et al, 2007. Novel substrates of *Mycobacterium tuberculosis* PknH Ser/Thr kinase. *Biochem. Biophys. Res. Commun.* 355, 162–168.

Article Annexe I:

From the characterization of the four serine/threonine protein kinases (PknA/B/G/L) of *Corynebacterium glutamicum* toward the role of PknA and PknB in cell division

Fiuza M, **Canova MJ**, Zanella-Cléon I, Becchi M, Cozzone AJ, Mateos LM, Kremer L, Gil JA, Molle V

Journal of Biological Chemistry. 2008,283:18099-112

Article Annexe II:

The MurC ligase essential for peptidoglycan biosynthesis is regulated by the serine/threonine protein kinase PknA in *Corynebacterium glutamicum*

Fiuza M, **Canova MJ**, Patin D, Letek M, Zanella-Cléon I, Becchi M, Mateos LM, Mengin-Lecreulx D, Molle V, Gil JA

Journal of Biological Chemistry 2008, 283:36553-63

Présentation des articles Annexes I et II :

Corynebacterium glutamicum fait partie de la famille des actinomycètes, et correspond à un organisme très étudié notamment par le fait d'être de la même famille que *M. tuberculosis* et donc de servir d'outil à l'étude de certains gènes mycobactériens. De plus, c'est un organisme abondamment exploité dans l'industrie agro-alimentaire, notamment pour la production de L-lysine et de L-glutamate.

L'analyse *in silico* du génome de cette bactérie a permis de prédire l'existence de quatre STPKs nommées PknA, PknB, PknG et PknL.

Dans cette étude, nous présentons la première caractérisation biochimique des quatre STPKs de *C. glutamicum* ainsi que leur rôle potentiel dans le contrôle de la forme de la cellule et de la synthèse du peptidoglycane durant la division cellulaire.

Des tests *in vitro* nous ont, tout d'abord, permis de démontrer que les différentes STPKs, à l'exception de PknG, possédaient une activité d'autophosphorylation. Des analyses par spectrométrie de masse ont ensuite permis d'identifier les sites spécifiques de phosphorylation de chaque kinase, montrant ainsi une variation dans le nombre, la nature et la localisation des sites phosphoaccepteurs entre les différentes STPKs.

Enfin, afin de mettre en évidence le rôle physiologique de chacune des kinases corynebactériennes, une approche par interruption de gène a permis de démontrer le caractère essentiel de *pknA* et *pknB*, alors que *pknG* et *pknL* n'apparaissent pas comme des gènes essentiels à la croissance et au développement de *C. glutamicum*. En effet, plusieurs expériences de délétion partielle et de surexpression de *pknA* et *pknB* *in vivo* ont montré que les deux kinases correspondantes jouent un rôle clé dans la régulation de la forme de la cellule ainsi que dans la division cellulaire.

Suite à l'étude des quatre STPKs corynebactériennes, nous avons également identifié la protéine MurC, un facteur impliqué dans la synthèse du peptidoglycane, comme correspondant à un substrat spécifique de PknA. Six sites de phosphorylation ont pu être identifiés par spectrométrie de masse. Par ailleurs, afin d'appréhender le rôle de la phosphorylation dans la régulation de l'activité de MurC, des mutants Ala ont été générés par mutagenèse dirigée en remplacement des six résidus Thr. Des tests d'activité *in vitro* et *in vivo* ont ensuite été réalisés avec les mutants ainsi générés, montrant une activité catalytique diminuée pour les mutants en comparaison de la protéine MurC sauvage. Egalement, d'autres tests *in vitro* ont révélé une activité catalytique moindre des formes phosphorylées de MurC en comparaison des formes non phosphorylées, mettant ainsi en évidence le rôle de régulation négative de la phosphorylation sur l'activité propre de cette protéine.

VOLUME 282 (2007) PAGES 14121–14131
DOI 10.1074/jbc.A611728200

***Arabidopsis* phosphatidylinositol phosphate kinase 1 binds F-actin and recruits phosphatidylinositol 4-kinase β 1 to the actin cytoskeleton.**

Amanda J. Davis, Yang Ju Im, Joshua S. Dubin, Kenneth B. Tomer, and Wendy F. Boss

This article has been retracted at the request of the authors as some of the results cannot be reproduced.

VOLUME 283 (2008) PAGES 18099–18112
DOI 10.1074/jbc.A802615200

From the characterization of the four serine/threonine protein kinases (PknA/B/G/L) of *Corynebacterium glutamicum* toward the role of PknA and PknB in cell division.

Maria Fiuza, Marc J. Canova, Isabelle Zanella-Cléon, Michel Becchi, Alain J. Cozzone, Luis M. Mateos, Laurent Kremer, José A. Gil, and Virginie Molle

On page 18099, the Ministerio de Ciencia y Tecnología grant number should be BIO2008-00519 instead of BIO2008-03234.

VOLUME 284 (2009) PAGES 363–371
DOI 10.1074/jbc.A808126200

Rnd1 regulates axon extension by enhancing the microtubule destabilizing activity of SCG10.

Ying-Hua Li, Sharang Ghavampour, Percy Bondallaz, Lena Will, Gabriele Grenningloh, and Andreas W. Püschel

Dr. Ghavampour's name was misspelled. The correct spelling is shown above.

VOLUME 284 (2009) PAGES 11100–11109
DOI 10.1074/jbc.A806956200

Role of Ca^{2+} /calmodulin-dependent protein kinase II in *Drosophila* photoreceptors.

Haiqin Lu, Hung-Tat Leung, Ning Wang, William L. Pak, and Bih-Hwa Shieh
Dr. Leung's present address is now Grambling State University, Grambling, LA 71245.

VOLUME 282 (2007) PAGES 29230–29240
DOI 10.1074/jbc.A703461200

Sorafenib functions to potently suppress RET tyrosine kinase activity by direct enzymatic inhibition and promoting RET lysosomal degradation independent of proteasomal targeting.

Iván Plaza-Menacho, Luca Mologni, Elisa Sala, Carlo Gambacorti-Passerini, Anthony I. Magee, Thera P. Links, Robert M. W. Hofstra, David Barford, and Clare M. Isacke

On page 29239 under "Discussion," the citation of Fig. 5 in line 17 should be changed to Fig. 7, and that of Fig. 6 in line 25 should be changed to Fig. 8.

VOLUME 284 (2009) PAGES 10808–10817
DOI 10.1074/jbc.A808333200

JDP2 (Jun dimerization protein 2)-deficient mouse embryonic fibroblasts are resistant to replicative senescence.

Koji Nakade, Jianzhi Pan, Takahito Yamasaki, Takehide Murata, Bohdan Wasyluk, and Kazunari K. Yokoyama

On page 10808, in addition to the grants listed, this work was also supported by Kaohsiung Medical University Grant KMU-EM-98-3 (to K. K. Y.).

We suggest that subscribers photocopy these corrections and insert the photocopies in the original publication at the location of the original article. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.

From the Characterization of the Four Serine/Threonine Protein Kinases (PknA/B/G/L) of *Corynebacterium glutamicum* toward the Role of PknA and PknB in Cell Division*

Received for publication, April 3, 2008, and in revised form, April 24, 2008. Published, JBC Papers in Press, April 28, 2008, DOI 10.1074/jbc.M802615200

Maria Fiuza^{†1}, Marc J. Canova[§], Isabelle Zanella-Cléon[§], Michel Becchi[§], Alain J. Cozzone[§], Luís M. Mateos[‡], Laurent Kremer[¶], José A. Gil[‡], and Virginie Molle^{§2}

From the [‡]Departamento de Biología Molecular, Área de Microbiología, Facultad de Biología, Universidad de León, León 24071, Spain, the [§]Institut de Biologie et Chimie des Protéines, UMR 5086, CNRS, Université Lyon 1, IFR128 BioSciences, Lyon-Gerland, 7 Passage du Vercors, 69367 Lyon Cedex 07, France, the [¶]Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, Université de Montpellier II et I, CNRS, UMR 5235, Case 107, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France, and ^{||}INSERM, DIMNP, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France

Corynebacterium glutamicum contains four serine/threonine protein kinases (STPKs) named PknA, PknB, PknG, and PknL. Here we present the first biochemical and comparative analysis of all four *C. glutamicum* STPKs and investigate their potential role in cell shape control and peptidoglycan synthesis during cell division. *In vitro* assays demonstrated that, except for PknG, all STPKs exhibited autokinase activity. We provide evidence that activation of PknG is part of a phosphorylation cascade mechanism that relies on PknA activity. Following phosphorylation by PknA, PknG could transphosphorylate its specific substrate OdhI *in vitro*. A mass spectrometry profiling approach was also used to identify the phosphoresidues in all four STPKs. The results indicate that the nature, number, and localization of the phosphoacceptors varies from one kinase to the other. Disruption of either *pknL* or *pknG* in *C. glutamicum* resulted in viable mutants presenting a typical cell morphology and growth rate. In contrast, we failed to obtain null mutants of *pknA* or *pknB*, supporting the notion that these genes are essential. Conditional mutants of *pknA* or *pknB* were therefore created, leading to partial depletion of PknA or PknB. This resulted in elongated cells, indicative of a cell division defect. Moreover, overexpression of PknA or PknB in *C. glutamicum* resulted in a lack of apical growth and therefore a coccoid-like morphology. These findings indicate that *pknA* and *pknB* are key players in signal transduction pathways for the regulation of the cell shape and both are essential for sustaining corynebacterial growth.

Corynebacterium glutamicum is a leading industrial amino acid producer and a model organism of the Corynebacteriaceae, a suborder of the actinomycetes that also includes the

genus *Mycobacterium*. This soil-borne, nonpathogenic Gram-positive actinomycete, which is widely used in the industrial production of amino acids, such as L-lysine and L-glutamic acid (1), has been extensively studied leading to the development of efficient genetic manipulation systems (3).

The genetics of cell growth and cell division of *C. glutamicum* started even before the complete genome sequence was available. The earliest studies focused on the sequencing and characterization of corynebacterial genes present in the conserved division and cell wall cluster (2). Once the genome sequence was available, it was evident that this bacterium, as well as different members of the actinomycetes, was deficient in many essential genes for cell division (3) and therefore corresponded to a minimalist version of a more sophisticated cell division apparatus (divisome) present in other bacteria. For instance, *C. glutamicum* is lacking genes homologue to *ftsA* (an actin homologue), to positive regulators involved in FtsZ polymerization such as *zipA* or *zapA*, or to negative regulators such as *ezrA*, *noc*, *slmA*, *sulA*, and *minCD* (3). Moreover, several essential cell division genes (*i.e.* *ftsN* and *ftsL*) are absent in *C. glutamicum*. Unlike other bacterial models, peptidoglycan (PG)³ biosynthesis in corynebacteria occurs at the septum and the cell poles (4, 5) and not at the lateral cell wall like in *Bacillus subtilis* or *Escherichia coli*. With respect to PG biosynthesis, *C. glutamicum* possesses only nine penicillin-binding proteins, in contrast to *E. coli* or *B. subtilis* that contain 13 and 16 penicillin-binding proteins, respectively (6). The mechanism by which *C. glutamicum* shifts between synthesis of PG at mid-cell for cell division or at the cell poles for cell elongation remains unclear, although this process is likely to be regulated in response to different stimuli encountered during growth.

Protein phosphorylation is a key mechanism by which environmental signals are transmitted to control protein activities in both eukaryotic and prokaryotic cells. Three main super-families of protein kinases are described as follows, the first one includes the “eukaryotic like” serine/threonine protein kinases

* This work was supported in part by grants from the Region Rhone-Alpes (to M. C.), the CNRS, the University of Lyon (France), National Research Agency Grant ANR-06-MIME-027-01 (to V. M. and L. K.), Junta de Castilla y León Grant LE040A07 (to J. A. G.), and Ministerio de Ciencia y Tecnología (Spain) Grants BIO2008-03234 and BIO2005-02723 (to J. A. G. and L. M. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Recipient of a fellowship from the Ministerio de Educación y Ciencia.

² To whom correspondence should be addressed. Tel.: 33-4-72-72-26-79; Fax: 33-4-72-72-26-41; E-mail: vmolle@ibcbp.fr.

³ The abbreviations used are: PG, peptidoglycan; STPK, serine/threonine protein kinase; GST, glutathione S-transferase; MS/MS, tandem mass spectrometry; DAPI, 4',6-diamino-2-phenylindole; PASTA, penicillin-binding protein and serine/threonine kinase-associated; Ni-NTA, nickel-nitrilotriacetic acid; TEV, tobacco etch virus; Van-FL, fluorescent vancomycin.

Serine/Threonine Protein Kinases from *C. glutamicum*

TABLE 1

Bacterial strains and plasmids used in this study

The abbreviation used is as follows: nt, nucleotide.

Strains or plasmids	Genotype or description	Source or Ref.
<i>E. coli</i> TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>endA1</i> <i>nupG</i> ; used for general cloning	Invitrogen
<i>E. coli</i> S17-1	Mobilizing donor strain, <i>pro</i> <i>recA</i> , which possesses an RP4 derivative integrated into the chromosome	62
<i>E. coli</i> BL21(DE3)Star	F2 <i>ompT</i> <i>hsdSB</i> (rB2 mB2) <i>gal</i> <i>dcm</i> (DE3); used to express recombinant proteins in <i>E. coli</i>	Stratagene
<i>C. glutamicum</i> ATCC 13869	Wild type control strain	ATCC
<i>C. glutamicum</i> R31	<i>C. glutamicum</i> ATCC 13869; derivative used as recipient in conjugations	63
pGEM-TEasy	<i>E. coli</i> vector; <i>bla</i> <i>lacI</i> <i>oriF1</i>	Promega
pK18mob	Mobilizable plasmid containing a <i>E. coli</i> origin of replication and <i>kan</i> resistance gene	64
pKAint	pK18mob derivative carrying an internal 485-bp fragment of <i>pknA</i> from <i>C. glutamicum</i>	This work
pKBint	pK18mob derivative carrying an internal 502-bp fragment of <i>pknB</i> from <i>C. glutamicum</i>	This work
pKGint	pK18mob derivative carrying an internal 849-bp fragment of <i>pknG</i> from <i>C. glutamicum</i>	This work
pKLint	pK18mob derivative carrying an internal 754-bp fragment of <i>pknL</i> from <i>C. glutamicum</i>	This work
pKLA	pK18mob derivative carrying the 5'-end (472 nt) of <i>pknA</i> from <i>C. glutamicum</i> under control of the P _{lac} promoter	This work
pKLB	pK18mob derivative carrying the 5'-end (470 nt) of <i>pknB</i> from <i>C. glutamicum</i> under control of the P _{lac} promoter	This work
pKPD	pK18mob derivative carrying the 5'-end (550 nt) of <i>divIVA</i> under the control of the <i>C. glutamicum</i> <i>gntK</i> gene promoter (P _{gntK} -Δ <i>divIVA</i> _{CG})	2
pKPKA	pKPD derivative carrying the 5'-end (472 nt) of <i>pknA</i> under the control of the <i>C. glutamicum</i> <i>gntK</i> gene promoter (P _{gntK} -Δ <i>pknA</i> _{CG})	This work
pKPKB	pKPD derivative carrying the 5'-end (470 nt) of <i>pknB</i> under the control of the <i>C. glutamicum</i> <i>gntK</i> gene promoter (P _{gntK} -Δ <i>pknB</i> _{CG})	This work
pECM2	Mobilizable plasmid able to replicate in <i>E. coli</i> and <i>C. glutamicum</i> ; <i>kan</i> and <i>cat</i> resistance genes	65
pEDiv	pECM2 derivative carrying P <div></div>	A. Ramos, unpublished data
pECDA	pEDiv derivative carrying P <div>-<i>pknA</i></div>	This work
pECDB	pEDiv derivative carrying P <div>-<i>pknB</i></div>	This work
pECDG	pEDiv derivative carrying P <div>-<i>pknG</i></div>	This work
pECDL	pEDiv derivative carrying P <div>-<i>pknL</i></div>	This work
pGEX4T-3	<i>E. coli</i> vector designed to make GST gene fusions	GE Healthcare
pGEXA	pGEX4T-3 derivative used to express GST fusion of PknA cytoplasmic domain	This work
pGEXB	pGEX4T-3 derivative used to express GST fusion of PknB cytoplasmic domain	This work
pGEXL	pGEX4T-3 derivative used to express GST fusion of PknL cytoplasmic domain	This work
pETTev	pET15b (Novagen) derivative that includes the replacement of the thrombin site coding sequence with a TEV protease site	21
pTEVGfull	pETTev derivative used to express His-tagged PknG	This work
pTEVOdhI	pETTev derivative used to express His-tagged OdhI	This work

(STPKs) (7–9); the second one corresponds to the newly characterized prokaryotic class of tyrosine kinases (10); and the third one corresponds to the well known family of bacterial kinases, the sensor histidine kinases, which are key enzymes of the so-called “two-component systems” (11). In bacteria, the presence of several STPKs suggests a central role of protein phosphorylation in regulating various biological functions, ranging from environmental adaptive responses to bacterial pathogenicity, like in the actinomycete relative *Mycobacterium tuberculosis* (12, 13). Thus, regulatory devices involving STPKs and phosphatases represent an emerging theme in prokaryotic signaling cascades. We therefore addressed the question whether STPKs could be involved in the model of growth in *C. glutamicum*.

In silico analysis of the genome sequence of *C. glutamicum* predicted the presence of four different STPKs (14). Three of these appear to be transmembrane proteins, with a putative extracellular signal sensor domain and an intracellular kinase domain. One of them, PknG, presented as a soluble kinase, has been investigated for its physiological role (15). Recent studies have demonstrated that in *M. tuberculosis* the STPKs PknA and PknB regulate cell growth (16) and cell division (17, 18). Because of the conserved genetic organization of the *pknA/pknB* cluster in *M. tuberculosis* and *C. glutamicum*, it is tempting to speculate that cell shape and division are also regulated by phosphorylation in *C. glutamicum*.

In this study, we focused on the four putative STPKs of *C. glutamicum*. As a first step in deciphering the potential role/participation of these kinases in the cell division process, the four proteins were characterized biochemically through a combination of *in vitro* phosphorylation assays and mass spectrometric analysis of their phosphorylation sites. We also demonstrated that either overexpression or depletion of PknA and PknB in *C. glutamicum* causes major growth and morphological changes, very likely resulting from cell division and cell wall biosynthesis alterations.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions, and Conjugal Plasmids—Bacterial strains and plasmids are described in Table 1. Strains used for cloning and expression of recombinant proteins were *E. coli* TOP10 (Invitrogen) and *E. coli* BL21(DE3)Star (Stratagene), respectively. *E. coli* cells were grown and maintained at 37 °C in LB medium supplemented with 100 μg/ml ampicillin and/or 50 μg/ml kanamycin, when required. *C. glutamicum* cells were grown at 30 °C in TSB (tryptic soy broth, Oxoid) or TSA (TSB containing 2% agar) medium supplemented with 12.5 μg/ml kanamycin. Plasmids to be transferred by conjugation from *E. coli* to *C. glutamicum* were introduced by transformation into the donor strain *E. coli* S17-1. Mobilization of plasmids from *E. coli* to coryneform strains was accomplished as described previously (19).

TABLE 2
Primers used in this study

Primer	Gene	5' to 3' sequence ^a
pknAint1	<i>pknA</i>	GCTCTAGAGCGCGAAGGCAGACTG (XbaI)
pknAint2	<i>pknA</i>	TATGGATCCCTGCGCAACGA (BamHI)
pknBint1	<i>pknB</i>	CCGGAATTCACGATTCACCTCCGCC (EcoRI)
pknBint2	<i>pknB</i>	TATGGATCCAAGAAGACCCTGACC (BamHI)
pknGint1	<i>pknG</i>	CCGGAATTCCTCCCTCCCTGAAAGAC (EcoRI)
pknGint2	<i>pknG</i>	TCCTAAGCTTCTTCGGTGGTAAAG (HindIII)
pknLint1	<i>pknL</i>	CCGGAATTCGATTCCTGGAGAGTTTTTG (EcoRI)
pknLint2	<i>pknL</i>	TCCTAAGCTTCATCGTCAGAATATTCACC (HindIII)
pknAlac1	<i>pknA</i>	CCGGAATTCATGAGTCAAGAAGACATCCTG (EcoRI)
pknAlac2	<i>pknA</i>	GCAAGCTTGTGATCAGCATGTTGCCCG (HindIII)
pknBlac1	<i>pknB</i>	CCGGAATTCCTGACCTTCGTGATCGCT (EcoRI)
pknBlac2	<i>pknB</i>	GCTCTAGAGCCGAGTCCATGACTTTCAC (XbaI)
pknA1	<i>pknA</i>	GGAATTCATATGAGTCAAGAAGACATCAC (NdeI)
pknAK	<i>pknA</i>	CTTTTTTTTTTAAAGTGATCAGCATGTTGCCCG (DraI)
pknB1	<i>pknB</i>	GGAATTCATATGACCTTCGTGATCGCT (NdeI)
pknBK	<i>pknB</i>	TGATATCGCCGAAGTCCATGACTTTCAC (EcoRV)
pknA2	<i>pknA</i>	GGAATTCATATGTCACCTGCGCTCCCTCA CAC (NdeI)
pknB2	<i>pknB</i>	GGAATTCATATGCTATTGACAGAGTGCAGCGA (NdeI)
pknG1	<i>pknG</i>	GGAATTCATATGAAGGATAATGAAGATTTTC (NdeI)
pknG2	<i>pknG</i>	GGAATTCATATGCTAGAACCAACTCAG (NdeI)
pknL1	<i>pknL</i>	GGAATTCATATGGCAAACCTGAAGGTCG (NdeI)
pknL2	<i>pknL</i>	GGAATTCATATGCTAAACGCCCTTACTG (NdeI)
pknAcd1	<i>pknA</i>	CCGGAATTCATGAGTCAAGAAGACATCAC (EcoRI)
pknAcd2	<i>pknA</i>	TATATTCTCGAGTCACAATGCCGGAACC (XhoI)
pknBcd1	<i>pknB</i>	TATGGATCCACCTTCGTGATCGCTGATCGC (BamHI)
pknBcd2	<i>pknB</i>	TATAAGCTTCTAGGCCACTTGGGTGGAGGTGCG (HindIII)
pknLcd1	<i>pknL</i>	CGGGATCCATGGCAAACCTGAAGGTC (BamHI)
pknLcd2	<i>pknL</i>	TATATTCTCGAGCTAAATTGACCACAACGTCAG (XhoI)
pknGfull2	<i>pknG</i>	TAATAGCTGCTAGCCTAGAACCAACTCAGTGGCCG (NheI)
534	<i>odhI</i>	TAATAGCTCATATGAGCGACAACAACGGCACCCCG (NdeI)
535	<i>odhI</i>	TAATAGCTGCTAGCTTACTCAGCAGGCCCTGCGAGGAAAAC (NheI)
M13-26		CAGGAAACAGCTATGAC

^a Restriction sites are underlined and indicated in parentheses.

Cloning, Expression, and Purification of PknA, PknB, PknL, and PknG—PCR fragments encoding the cytoplasmic domain corresponding to the kinase domain and the juxtamembrane linker of PknA (residues 1–332 out of 469), PknB (residues 1–313 out of 646), and PknL (residues 1–396 out of 740) were amplified by using *C. glutamicum* ATCC 13869 genomic DNA as a template. The different *pkn* gene fragments coding for the N-terminal cytoplasmic region, with appropriate sites at both ends, were synthesized by PCR amplification using the primer pairs pknAcd1/pknAcd2, pknBcd1/pknBcd2, and pknLcd1/pknLcd2, respectively (Table 2). These DNA fragments were digested with the corresponding restriction enzymes and ligated into vector pGEX4T-3 (see Table 1), digested with the same enzymes, to yield pGEXA, pGEXB, and pGEXL, respectively.

E. coli BL21(DE3)Star cells were transformed with the pGEX4T-3 vector derivatives expressing the PknA_{CD}, PknB_{CD}, and PknL_{CD} proteins. Recombinant *E. coli* strains harboring the pGEX4T-3 derivatives were used to inoculate 200 ml of LB medium supplemented with ampicillin and incubated at 37 °C with shaking until A_{600} reached 0.5. Isopropyl 1-thio- β -D-galactopyranoside was then added at a final concentration of 0.5 mM, and growth was continued for an additional 3-h period at 30 °C. Recombinant GST kinase cytoplasmic regions were purified on glutathione-Sepharose 4B matrix as described previously (20).

For the cloning, expression, and purification of the cytoplasmic PknG recombinant protein (822 residues), the full-length *pknG* gene was amplified by PCR using *C. glutamicum* ATCC 13869 genomic DNA as a template and the primer pair pknG1 and pknGfull2 (Table 2). This PCR product was digested with

the restriction enzymes corresponding to the appropriate sites at both ends and ligated into pETTeV (Table 1), the variant of pET15b (Novagen) that includes the replacement of the thrombin site coding sequence with a tobacco etch virus (TEV) protease site (21), yielding pTEVGfull. *E. coli* BL21(DE3)Star cells transformed with this construction were used for expression and purification of His-tagged PknG as described previously (22).

Construction and Purification of OdhI Recombinant Protein—The *odhI* gene was amplified by PCR using *C. glutamicum* ATCC 13869 genomic DNA as a template and the primer pair 534/535, containing an NdeI and NheI restriction site, respectively. This 432-bp amplified product was digested by NdeI and NheI, and ligated into pETTeV (Table 1) generating the pTEVodhI. *E. coli* BL21(DE3)Star cells transformed with this construct were used for expression and purification of His-tagged OdhI as described previously (22). Finally, the purified His-tagged OdhI was treated with TEV protease according to the manufacturer's instructions (Invitrogen).

Gene Inactivation in C. glutamicum—Internal fragments of the *pknA*, *pknB*, *pknG*, and *pknL* genes were amplified from the *C. glutamicum* ATCC 13869 chromosome using pknAint1/pknAint2, pknBint1/pknBint2, pknGint1/pknGint2, and pknLint1/pknLint2 primer pairs (Table 2), respectively. The amplified 485-, 502-, 849-, and 754-bp DNA fragments were purified and cloned into pGEM-TEasy (Table 1) and later were digested and subcloned into the conjugative suicide plasmid pK18mob (Table 1), yielding to plasmids pKAint, pKBint, pKGint, and pKLint, respectively (Table 1). All these plasmids were used to disrupt the *C. glutamicum pkn* genes by single recombination. The corresponding DNA inserts were used as probes in South-

Serine/Threonine Protein Kinases from *C. glutamicum*

ern blot experiments to confirm gene disruption. Total DNA from corynebacteria was isolated using the Kirby method described for *Streptomyces* (23), except that the cells were treated with lysozyme for 4 h at 30 °C. For Southern blot analysis, genomic DNA from different *C. glutamicum* transconjugants were digested with different restriction enzymes and loaded on agarose gels. Samples were transferred to nylon membranes and hybridized with digoxigenin-labeled probes according to the manufacturer's instructions (Roche Applied Science) and conventional protocols. To reduce the PknA or PknB levels in *C. glutamicum*, two sets of plasmids were designed to express either *pknA* or *pknB* under the control of P_{lac} and P_{gntK} promoters. Plasmids pKLA and pKLB were constructed as follows. The first 472-bp *pknA* fragment or the 470-bp *pknB* fragment was amplified by PCR using pknAlac1/pknAlac2 and pknBlac1/pknBlac2, respectively (Table 2), digested with EcoRI and HindIII/XbaI, and subcloned into the EcoRI-HindIII/XbaI sites of pK18mob (Table 1). To place *pknA* or *pknB* under the control of the regulated promoter P_{gntK} (promoter of the *gntK* gene encoding the gluconate kinase from *C. glutamicum*) (2), the 5'-ends of *pknA* (472 bp) and *pknB* (470 bp) were PCR-amplified using the pknA1/pknAK and pknB1/pknAK primer pairs, respectively (Table 2), digested with NdeI and DraI/EcoRV, and subcloned into NdeI/EcoRI (Klenow-filled) digested plasmid pKPD (Table 1), yielding to plasmids pKPKA and pKPKB, respectively. To study the expression of the four *pkn* genes in *C. glutamicum* under the control of the strong promoter P_{div} (promoter of the *divIVA* gene from *C. glutamicum*) (5, 24), plasmids pECDA/B/G/L were constructed as follows: *pknA/B/G/L* were PCR-amplified using the primer pairs pknA1/pknA2, pknB1/pknB2, pknG1/pknG2, or pknL1/pknG2, digested with NdeI, and subsequently cloned under the control of the P_{div} promoter into plasmid pEDiv (Table 1).

Microscopic Techniques—Living *C. glutamicum* cells or cells stained with fluorescent dyes were observed in a Nikon E400 phase contrast and fluorescence microscope. Pictures were taken with a DN100 Nikon digital camera. Vancomycin BODIPY-FL (VAN-FL) (Molecular Probes) staining was performed by adding an equal proportion of unlabeled vancomycin and Van-FL to growing cultures at a final concentration of 1 $\mu\text{g}/\text{ml}$ (4). After incubation for 5 min to allow absorption of the antibiotic, cells were directly observed by fluorescence microscopy. Staining with 4',6-diamino-2-phenylindole (DAPI) was performed as described previously (25).

In Vitro Kinase Assays—*In vitro* phosphorylation of each recombinant kinase (0.5 μg) was carried out for 30 min at 37 °C in a reaction mixture (20 μl) containing buffer P (25 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol, 5 mM MgCl_2 , 1 mM EDTA) with 200 $\mu\text{Ci}/\text{ml}$ [γ - ^{33}P]ATP corresponding to 65 nM (3000 Ci/mmol) (PerkinElmer Life Sciences). After incubation, the reaction was stopped by adding sample buffer and heating the mixture at 100 °C for 5 min. The reaction mixtures were analyzed by SDS-PAGE. After electrophoresis, gels were soaked in 20% trichloroacetic acid for 10 min at 90 °C, stained with Coomassie Blue, and dried. Radioactive proteins were visualized by autoradiography using direct exposure films. For mass spectrometry analysis, kinases were phosphorylated as described above, excepted that [γ - ^{33}P]ATP was replaced with 5 mM ATP.

Sample Preparation and Mass Spectrometry Analysis—Purified GST-PknA_{CD}, GST-PknB_{CD}, GST-PknL_{CD}, and His-PknG were subjected to *in vitro* phosphorylation with nonradioactive ATP prior to resolving on SDS-PAGE. In-gel digestion was performed as described by Shevchenko *et al.* (26) with minor modifications. Samples were reduced with 60 μl of 10 mM dithiothreitol in 50 mM NH_4HCO_3 for 40 min at 56 °C. Alkylation was performed with 60 μl of 55 mM iodoacetamide in 50 mM NH_4HCO_3 for 30 min at room temperature in the dark. The gel pieces were successively dried, re-hydrated, and re-dried using 300 μl of CH_3CN , 200 μl of 50 mM NH_4HCO_3 , and 300 μl of CH_3CN , respectively. For proteolytic digestion, the protein-containing gel pieces were treated with 30–40 μl of trypsin (sequence grade, Promega, Madison, WI) for 45 min at 50 °C. A second extraction step was performed using 30 μl of $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{HCOOH}$ (60:36:4; v/v/v) mixture for 30 min at 30 °C, and finally all extracts were pooled and dried in a vacuum concentrator and resuspended in 0.1% trifluoroacetic acid (20 μl).

NanoLC/nanospray/tandem mass spectrometry experiments were performed on a Q-STAR XL instrument (QqTOF) (Applied Biosystems, Courtaboeuf, France) equipped with a nanospray source using a distal coated silica-tip emitter (FS 150-20-10-D-20, New Objective) set at 2300 V. By means of information-dependent acquisition mode, peptide ions within a m/z 400–2000 survey scan mass range were analyzed for subsequent fragmentation (three precursors). Double to quadruple charged ions exceeding a threshold of 10 counts were selected for MS/MS analyses. MS/MS spectra were acquired in the m/z 50–2000 range with a 30-s dynamic exclusion time. The collision energy was automatically set by the software (Analyst 1.1) and was related to the charge of the precursor ion. The MS and MS/MS data were recalibrated using internal reference ions from a trypsin autolysis peptide at m/z 842.510 [$\text{M} + \text{H}$]⁺ and m/z 421.759 [$\text{M} + 2\text{H}$]²⁺. Screening for phosphorylated peptides was achieved by the paragon method from the ProteinPilot® data base-searching software (version 2.0, Applied Biosystems). The LC part of the analytical system consisted of an LC-Packings nano-LC (Dionex, Voisins Le Bretonneux, France). Chromatographic separation of peptides was obtained in a C₁₈ PepMap micro-precolumn (5 μm ; 100 Å; 300 μm × 5 mm; Dionex) and a C₁₈ PepMap nano-column (3 μm ; 100 Å; 75 μm × 150 mm; Dionex). After injection (1- μl injection volume, pick-up mode, in a 20- μl injection loop), samples were adsorbed and desalted on the pre-column with a $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{trifluoroacetic acid}$ (98:2:0.05; v/v/v) solvent mixture for 3 min at 25 $\mu\text{l}/\text{min}$ flow rate. The peptide separation was developed using a linear 60-min gradient from 0 to 60% B, where solvent A was 0.1% HCOOH in $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (95/5) and solvent B was 0.1% HCOOH in $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (20/80) at \sim 200 nl/min flow rate.

RESULTS AND DISCUSSION

Genome Sequence Analysis of *C. glutamicum* for STPKs Encoding Genes—Examination of the *C. glutamicum* genome sequence revealed the presence of four putative genes encoding STPKs, all of which belong to the PKN2 family of prokaryotic protein kinases that are most closely related to the eukaryotic STPKs (27). To date, the corynebacterial STPKs have been

characterized to a limited extent. Only one of them, PknG, has been shown to participate in glutamine utilization (15), whereas the others (PknA, PknB, and PknL) remained uncharacterized. The genes encoding PknA and PknB are located, as in *M. tuberculosis* (16), in an apparent five-gene operon (*pstP*, *rodA*, *pbp2B*, *pknA*, and *pknB*). This operon is present in all corynebacterial genomes already sequenced (14, 28–30). The gene encoding PknL is present in the vicinity (6–8 kb) of the division and cell wall cluster comprising essential genes for cell division (*ftsQ* and *ftsZ*) and PG biosynthesis (*ftsI*, *murE*, *murF*, *mraY*, *murD*, *ftsW*, *murG*, and *murC*). PknL is in the opposite direction to the division and cell wall cluster, not only in corynebacteria, but also in other actinomycetes including *M. tuberculosis* (31, 32) and *Streptomyces coelicolor* (33). The gene encoding PknG is also located in a three-gene operon (*glnX*, *glnH*, and *pknG*) in all corynebacterial strains sequenced and, as mentioned above, is involved in glutamine utilization (15). Therefore, it seems essential to pursue the previous studies realized on the PknG kinase and also to newly characterize PknA, PknB, and PknL to extend our understanding of the role of corynebacterial STPKs.

Expression and Purification of the *C. glutamicum* STPKs—The *pknA*, *pknB*, *pknL*, and *pknG* genes encode 469-, 646-, 740-, and 822-amino acid proteins, with calculated molecular masses of 50, 68, 79, and 91 kDa, respectively. Primary sequence analysis revealed the presence of all the essential amino acids and sequence subdomains characterizing the Hanks' family of eukaryotic like protein kinases (34). These include the central core of the catalytic loop, consisting of subdomain VI, and the invariant lysine residue in the consensus motif within subdomain II, which is usually involved in the phosphotransfer reaction and required for the autophosphorylating activity of eukaryotic STPKs (Fig. 1) (34, 35). The hydropathy profiles of PknA, PknB, and PknL predict the presence of a single putative transmembrane constituting a membrane anchor (Fig. 2) (36). This transmembrane domain is lacking in PknG, which is a so-called cytoplasmic STPK. The extracellular portion of STPKs normally consists of one or more sensor domains that, when bound to their ligand, leads to intracellular conformational changes, which subsequently activate a signaling cascade. In some cases, this extracellular domain has been shown to possess penicillin-binding protein and serine/threonine kinase-associated (PASTA) motifs (37). In *M. tuberculosis* PknB, for instance, the extracellular portion contains four PASTA motifs, strongly supportive of a signal-binding sensor domain (38). This feature is also conserved in *C. glutamicum* PknB, which possesses three PASTA motifs (Fig. 2). Analysis of the *C. glutamicum* PknL primary sequence also predicts the presence of four PASTA motifs (Fig. 2), in contrast to *M. tuberculosis* PknL, which lacks the extracellular domain (31). This may imply that *C. glutamicum* PknL responds to different types of ligands than its mycobacterial homologue.

To allow detailed biochemical studies, we focused our attention on the N-terminal cytoplasmic domains of PknA, PknB, and PknL corresponding to residues 1–332 out of 469, residues 1–313 out of 646, and residues 1–396 out of 740, respectively, comprising the kinase domain as well as the juxtamembrane linker (Fig. 2). The truncated genes lacking the DNA segments

coding the hydrophobic C-terminal domain were synthesized by PCR amplification using genomic DNA from *C. glutamicum* ATCC 13869. The amplified products were cloned into plasmid pGEX4T-3, thus giving rise to pGEXA, pGEXB, and pGEXL. The recombinant GST-tagged PknA_{CD}, PknB_{CD}, and PknL_{CD} fusion proteins were expressed in *E. coli* and purified using a glutathione-Sepharose 4B matrix. Analysis of the GST chimeric proteins by SDS-PAGE revealed that the wild-type enzymes were expressed in a soluble form and migrated with their predicted molecular masses, but also showed the presence of extra bands (Fig. 3A, upper panel). All these bands were analyzed by mass spectrometry, which confirmed the identity of the GST chimeric proteins (data not shown). An aberrant migration property has been observed previously for *M. tuberculosis* kinases, such as PknD, PknE, PknL, and PknH (20, 31, 39, 40), as well as for Pkn9 and Pkn6, two *Myxococcus xanthus* STPKs (41, 42). It was demonstrated that this specific pattern of migration was because of the different phosphorylated isoforms. Taken together, these results indicate that PknA_{CD}, PknB_{CD}, and PknL_{CD} were produced as different soluble isoforms.

The full-length *pknG* gene (Fig. 2) was PCR-amplified using genomic DNA from *C. glutamicum* ATCC 13869. The product was cloned into plasmid pETTev, yielding to pTEVGfull. The recombinant His-tagged PknG protein was purified from *E. coli* using a Ni-NTA matrix. Analysis of the electrophoretic mobility of the protein by SDS-PAGE revealed that PknG was expressed in a soluble form according to its predicted molecular mass (Fig. 3A, upper panel).

Protein Kinase Activity of *C. glutamicum* STPKs—To investigate whether PknG, PknA_{CD}, PknB_{CD}, and PknL_{CD} display autokinase activity, the purified kinases were incubated individually with [γ -³³P]ATP as a phosphate donor, separated by SDS-PAGE, and exposed to a x-ray film. As shown in Fig. 3A (lower panel), PknA_{CD}, PknB_{CD}, and PknL_{CD} incorporated radioactive phosphate from [γ -³³P]ATP, generating a radioactive signal corresponding to the expected size of the protein isoforms, clearly indicating that these kinases undergo autophosphorylation. Unexpectedly, and in sharp contrast with PknA_{CD}, PknB_{CD}, or PknL_{CD}, PknG was not able to incorporate [γ -³³P]ATP (Fig. 3A, lower panel). To exclude the possibility that, in the full-length PknG protein, the C-terminal sensor domain could inhibit autophosphorylation activity, the PknG kinase domain alone was expressed, purified, and tested *in vitro* using [γ -³³P]ATP. No restoration of autophosphorylation activity could be detected, ruling out the hypothesis that the sensor domain exerts an inhibitory activity on PknG (data not shown).

Altogether, these data indicate that the N-terminal cytoplasmic domains of PknA, PknB, and PknL possess intrinsic autophosphorylation activity *in vitro*, whereas neither the kinase domain of PknG nor the full-length cytoplasmic protein showed autokinase activity. This prompted us to investigate whether cross-regulation/interaction between the PknG and PknA/B/L could occur.

Activation of PknG Phosphorylation Is Dependent on a PknA Cascade—In an elegant study, Kang *et al.* (16) demonstrated efficient intermolecular phosphorylation between PknA and PknB from *M. tuberculosis*, suggesting the possi-

Serine/Threonine Protein Kinases from *C. glutamicum*

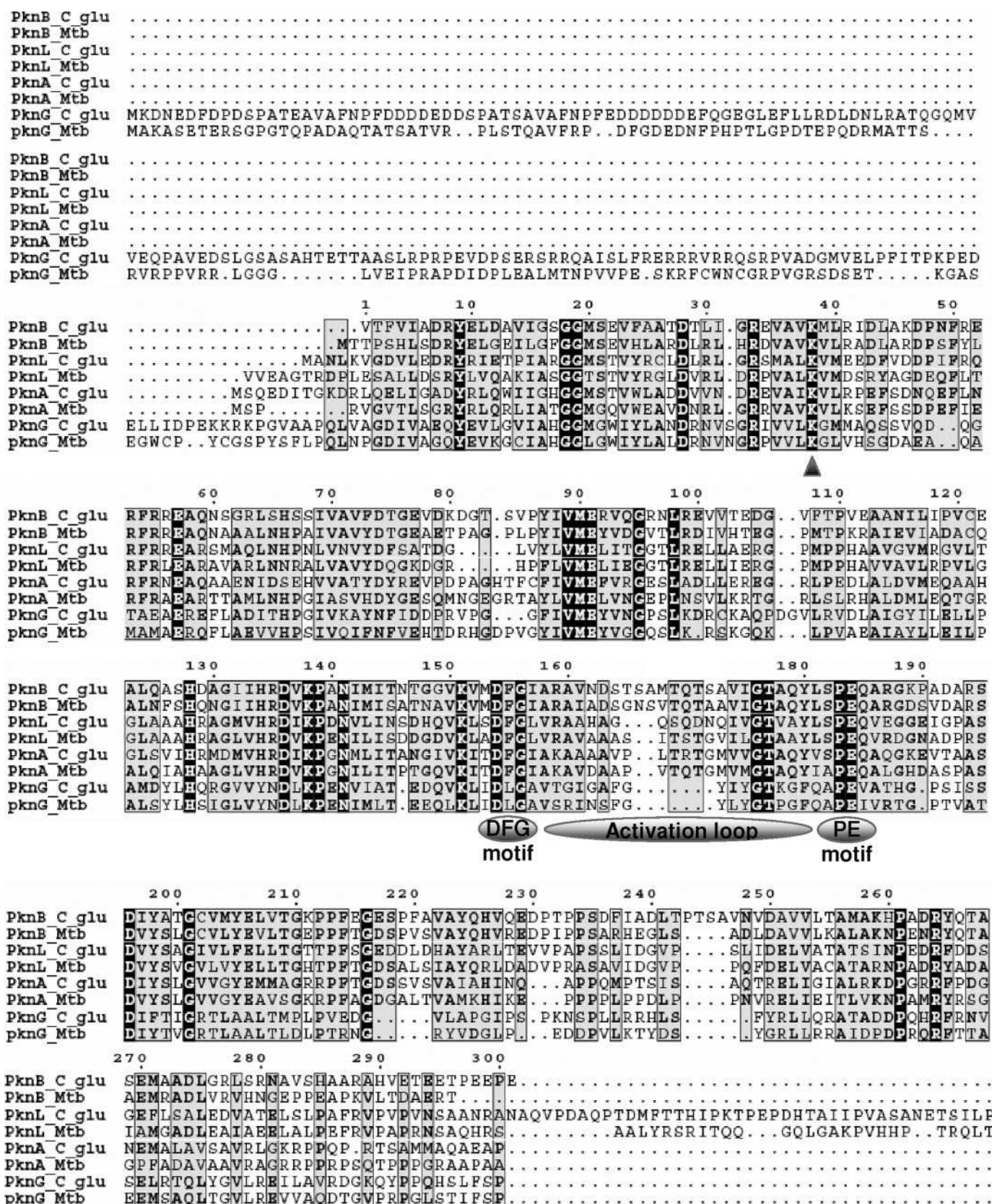


FIGURE 1. Multiple sequence alignment of the kinase domains of *C. glutamicum* STPKs with their respective *M. tuberculosis* homologues. The alignment was performed using ClustalW and Esprout programs (*C. glu.*, *C. glutamicum*; *Mtb.*, *M. tuberculosis*). The conserved activation loop of the STPK family, delimited by the DFG and PE motifs, is represented by shaded circles. The key lysine residue involved in binding ATP is indicated by a black triangle.

Downloaded from www.jbc.org at HARVARD UNIVERSITY on June 3, 2009

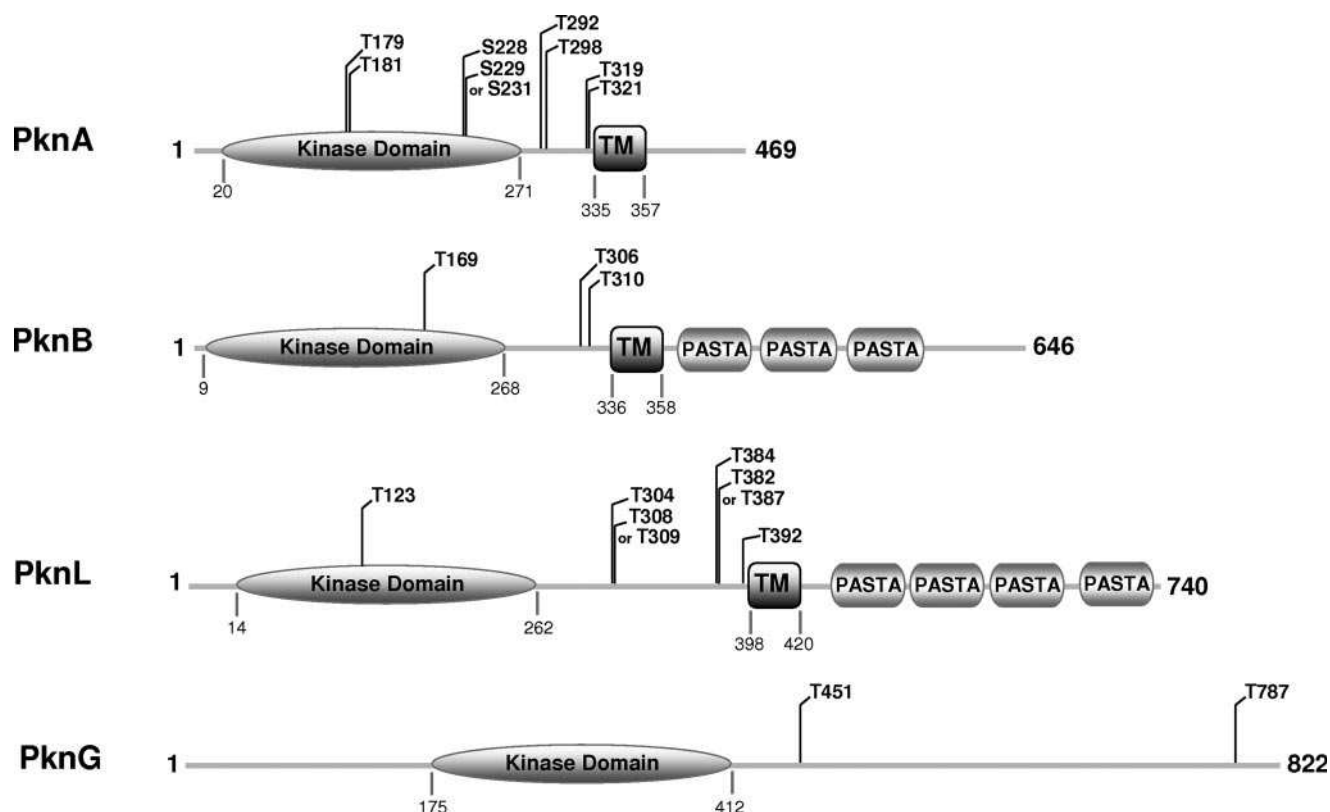


FIGURE 2. Phosphorylated residues in the cytoplasmic domains of the four *C. glutamicum* STPKs. The kinase domains and putative transmembrane regions (TM) are shown by shaded circles and shaded boxes, respectively. The extracellular PASTA motifs are represented by different blocks. Refer to Table 3 for further details.

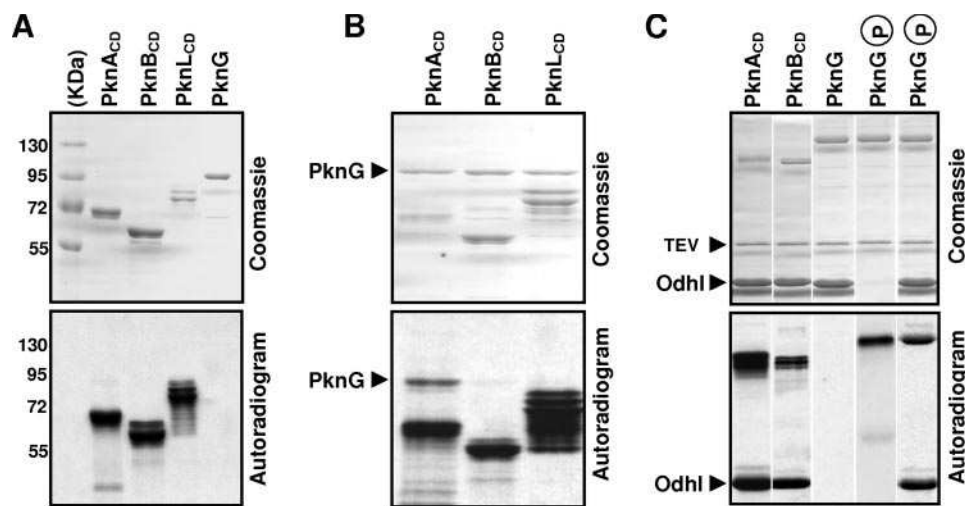


FIGURE 3. *In vitro* phosphorylation activity of *C. glutamicum* STPKs. *A*, cytoplasmic domains (CD) of PknA, PknB, and PknL proteins were overproduced and purified on glutathione-Sepharose 4B matrix. The full-length protein PknG was overproduced and purified on Ni-NTA matrix. The different kinases were submitted to gel electrophoresis and stained with Coomassie Blue (upper panel). *In vitro* phosphorylation assays were performed with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ for 30 min. Proteins were analyzed by SDS-PAGE, and radioactive bands were revealed by autoradiography (lower panel). Standard proteins of known molecular masses were run in parallel (kDa lane). *B*, *in vitro* phosphorylation of PknG with PknA_{CD}, PknB_{CD}, and PknL_{CD} was assayed with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ for 30 min. Proteins were analyzed by SDS-PAGE and stained with Coomassie Blue (upper panel), and radioactive bands were revealed by autoradiography (lower panel). *C*, *in vitro* transphosphorylation of Odhl by PknA_{CD}, PknB_{CD}, or PknG was assayed with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ for 30 min. Recombinant Odhl was treated with the TEV protease to remove the N-terminal His tag and used in phosphorylation assays in the presence of $[\gamma\text{-}^{33}\text{P}]\text{ATP}$: PknA_{CD} and Odhl (lane 1), PknB_{CD} and Odhl (lane 2), PknG and Odhl (lane 3), PknG after on-column phosphorylation by PknA (lane 4), and PknG after on-column phosphorylation by PknA and Odhl (lane 5). Proteins were separated by SDS-PAGE and stained with Coomassie Blue (upper panel), and radioactive bands were revealed by autoradiography (lower panel).

bility that cross-phosphorylation between these kinases may occur during signal transduction *in vivo*. This was demonstrated by assessing the ability of these kinases to phosphorylate each other, the wild-type form of one kinase mixed with the kinase-inactive form of the other (16).

We therefore reasoned that PknG may represent a substrate of PknA/B/L and that activation of PknG requires the protein to be phosphorylated by the other kinases. To test this hypothesis, His-tagged PknG was incubated with either PknA_{CD}, PknB_{CD}, or PknL_{CD} in the presence of $[\gamma\text{-}^{33}\text{P}]\text{ATP}$. As shown in Fig. 3B, PknA was able to phosphorylate PknG. This supports the notion that intermolecular phosphorylation between PknA and PknG occurs and therefore may be part of a complex phosphorylation cascade. However, in a recent study, Niebisch *et al.* (15) provided evidence that purified Strep-tagged PknG catalyzed autophosphorylation and transphosphorylation activity to its

Serine/Threonine Protein Kinases from *C. glutamicum*

specific substrate 2-oxoglutarate dehydrogenase inhibitor protein (OdhI). OdhI is a 15-kDa protein with a ForkHead-associated domain and a homologue of mycobacterial GarA. One possible explanation of the discrepancy between the two studies may rely on the origin of the PknG used. In their study, Niebisch *et al.* (15) utilized a kinase that was overexpressed and purified from *C. glutamicum*, whereas we used an *E. coli* recombinant PknG protein. It is very likely that PknG from *C. glutamicum* had already been phosphorylated *in vivo* by other kinases, presumably PknA and “activated” to recognize and phosphorylate OdhI *in vitro*. However, whether PknG is phosphorylated by either PknA, PknB, or both PknA/B *in vivo* remains challenging, because null mutants of either *pknA* or *pknB* are lethal (see below), thus rendering an analysis of the individual contribution of these two kinases in phosphorylating PknG extremely difficult.

It is noteworthy that a scenario where a kinase is part of complex signaling cascade involving other kinases has been reported earlier in *M. xanthus* where Pkn8, a membrane-associated STPK, phosphorylates Pkn14, a cytoplasmic STPK. This latter phosphorylates MrpC on Thr residues, a transcriptional regulator essential for *M. xanthus* development (43).

We next cloned, overexpressed, and purified the *C. glutamicum* OdhI protein from *E. coli* and used it in *in vitro* phosphorylation assays. OdhI was purified as a His-tagged protein, which was expressed in soluble form and migrated as a 15-kDa protein as judged by Coomassie Blue staining after separation by SDS-PAGE (Fig. 3C, upper panel). In a first set of experiments, aimed to delineate the network of interactions between PknA, PknB, PknG, and OdhI, we analyzed whether OdhI could serve as a substrate. Fig. 3C (lower panel) clearly shows that, unlike PknA or PknB (lanes 1 and 2), PknG was unable to phosphorylate OdhI (lane 3). In another set of experiments, we tested whether PknA-dependent phosphorylation of PknG (allowing activation of PknG) would allocate PknG to phosphorylate OdhI. Because PknG was expressed as a His-tagged protein and PknA as a GST fusion protein, the different tags allowed us to selectively resolve one kinase from the other. The Ni-NTA resin-bound PknG was incubated with [γ - 33 P]ATP and purified GST-PknA, thus allowing phosphorylation of the PknG kinase to occur. The Ni-NTA resin was extensively washed to eliminate the excess of unbound GST-PknA. Following elution from the Ni-NTA matrix, phosphorylated PknG was assayed *in vitro* with [γ - 33 P]ATP supplemented or not with OdhI (Fig. 3C, lanes 4 and 5). PknG could be efficiently phosphorylated by PknA (Fig. 3C, lane 4) and, because of the absence of radioactive signal corresponding to the appropriate size of GST-PknA, one can rule out the possibility of PknA contamination in the assay. The results indicate that PknG is required to be phosphorylated by PknA to efficiently transphosphorylate OdhI *in vitro* (Fig. 3C, lane 5). Overall, these findings are consistent with the hypothesis that PknG is dependent on PknA phosphorylation prior to phosphorylation of its specific substrate OdhI.

It is tempting to speculate that, *in vivo*, the PknA cascade is used to phosphorylate PknG, which in turn controls the 2-oxoglutarate dehydrogenase complex activity, a key enzyme of the tricarboxylic acid cycle, via the phosphorylation status of the

PknG substrate, the OdhI regulator protein (15). This mechanism is of particular importance for the biotechnology production of about 1.5 million tons/year of L-glutamate by coryneform bacteria, where attenuation of 2-oxoglutarate dehydrogenase activity was found to be the key factor in the metabolic network (44). Furthermore, considering that the proteins PknG, OdhI (named as GarA in *M. tuberculosis*), and the 2-oxoglutarate dehydrogenase (OdhA) are highly conserved between *C. glutamicum* and *M. tuberculosis*, the signaling cascade involving these proteins could be similar in mycobacteria. This assumption is supported by the fact that the intracellular glutamate/glutamine level measured in an *M. tuberculosis* $\Delta pknG$ strain is increased (45). However, the *M. tuberculosis* PknG kinase purified from *E. coli* was capable of autophosphorylation activity *in vitro* (46), thus indicating that these kinases could possess a different mechanism of activation/regulation in the two microorganisms. It is noteworthy that *M. tuberculosis* possesses 11 STPKs compared with only 4 in *C. glutamicum*; therefore, the mechanism of regulation could be slightly different because of the possibility of an important STPK cross-talk in *M. tuberculosis*. Although the OdhI homologue in *M. tuberculosis*, GarA, was originally found to be phosphorylated by PknB (47), it is possible that a regulation mechanism, including PknA, PknG, and GarA, occurs in *M. tuberculosis*, similarly to what happens in *C. glutamicum*. Overall, these results led us to hypothesize that, *in vivo*, PknG from *C. glutamicum* could be part of a phosphorylation cascade, which in turn activates its kinase activity to control the 2-oxoglutarate dehydrogenase complex. Whether the STPK cross-talk occurs *in vivo* in *C. glutamicum* remains to be established, however. Further studies are currently under way to characterize more precisely the phosphorylation network in *C. glutamicum*, which also requires the identification of the phosphorylation sites of the kinases.

Identification of the PknA_{CD}, PknB_{CD}, PknL_{CD}, and PknG Phosphorylation Sites—Characterization of the phosphorylation sites in proteins provides a powerful tool to study signal transduction pathways and to decipher interaction networks involving signaling elements and is therefore of high biological relevance. Thus, assignment of the phosphorylation sites in STPKs is a major challenge in proteomics, particularly because some of these *C. glutamicum* STPKs may be involved in cell division (PknA, PknB, and PknL) or in regulation of the tricarboxylic acid cycle (PknG). A mass spectrometry approach was therefore used to identify the phosphoresidues in all four *C. glutamicum* STPKs. This technique has recently been proven to be the method of choice for characterizing post-translational modifications such as phosphorylation (31, 48). NanoLC/nanospray/tandem mass spectrometry was applied for the identification of phosphorylated peptides and for localization of phosphorylation sites in the different kinases. Purified GST-PknA_{CD}, GST-PknB_{CD}, or GST-PknL_{CD} was subjected to *in vitro* phosphorylation with nonradioactive ATP, whereas His-tagged PknG was phosphorylated with PknA and nonradioactive ATP prior to resolving on SDS-PAGE and in-gel digestion with either trypsin or chymotrypsin. Peptides deriving from PknA_{CD}, PknB_{CD}, PknL_{CD}, or PknG were identified by nanoLC/nanospray/MS/MS, which led to 90, 90, 80, and 80% of

TABLE 3

Phosphorylation status of recombinant protein kinases PknA_{CD}, PknB_{CD}, PknL_{CD}, and PknG as determined by mass spectrometry

Phosphorylated tryptic and chymotryptic peptide sequence	Number of detected phosphate groups, LC-ESI/MS/MS ^a	Phosphorylated residue(s)
PknA_{CD}		
172 AAAAVPLTR ¹⁸⁰	1	Thr-179
172 AAAAVPLTRTGMVVGTAQYVSPEQAQGK ¹⁹⁹	1	Thr-179
172 AAAAVPLTRTGMVVGTAQYVSPEQAQGK ¹⁹⁹	2	Thr-179 and Thr-181
168 GIAKAAAAPLTRTGMVVGTAQY ¹⁹⁰	2	Thr-179 and Thr-181
222 RPFTGDSVSVVAIAHINQAPPQMPTSISAQTR ²⁵³	1	Ser-228 or Ser-229 or Ser-231
222 RPFTGDSVSVVAIAHINQAPPQMPTSISAQTR ²⁵³	2	Ser-228 and Ser-229 or Ser-231
225 TGDSSVVAIAH ²³⁶	1	Ser-228
283 LGKRPPQPRTSAMMAQAEAPSPSESTAMLGR ³¹³	2	Thr-292 and Thr-298
314 VARPATITQEAAPK ³²⁷	1	Thr-319
314 VARPATITQEAAPK ³²⁷	2	Thr-319 and Thr-321
314 VARPATITQEAAPK ³²⁷	1	Thr-321
317 PATITQEAAPK ³²⁷	2	Thr-319 and Thr-321
PknB_{CD}		
160 AVNDSTSAMTQTSAVIGTAQYLSPEQAR ¹⁸⁷	1	Thr-169
304 FSTRSTQVA ³¹³	1	Thr-306
304 FSTRSTQVA ³¹³	2	Thr-306 and Thr-310
PknL_{CD}		
120 GVLTGLAAHR ¹³⁰	1	Thr-123
294 ANAQPDAQPTDMFTTHIPK ³¹³	2	Thr-304 and (Thr-308 or Thr-309)
366 NIQDQELARADEPEINTVSNRSKL ³⁸⁹	1	Thr-382
375 ADEPEINTVSNR ³⁸⁶	1	Thr-382
375 ADEPEINTVSNRSK ³⁸⁸	2	Thr-382 and Ser-384 or Ser-387
389 LKLTLSWI ³⁹⁶	1	Thr-392
387 SKLKLTLWSI ³⁹⁶	1	Thr-392
PknG		
447 STFGTKHLVFR ⁴⁵⁷	1	Thr-451
450 GTKHLVF ⁴⁵⁶	1	Thr-451
784 GLRTGISEALR ⁷⁹⁴	1	Thr-787
782 SKGLRTGISEALR ⁷⁹⁴	1	Thr-787

^a LC-ESI/MS/MS is nanoLC/nanospray/tandem mass spectrometry.

sequence coverage, respectively. Therefore, one cannot exclude the possibility of additional phosphorylated serine or threonine residues not covered by this analysis. Phosphorylated amino acid residues were assigned by peptide fragmentation in MS/MS; *y* and *b* daughter ions containing one phosphoserine or phosphothreonine were associated with a neutral loss of phosphoric acid ($-H_3PO_4$, *i.e.* -98 Da).

As detailed in Table 3, and presented in Fig. 2, analysis of tryptic and chymotryptic digests allowed the characterization of 8, 3, 6, and 2 phosphorylation sites in PknA_{CD}, PknB_{CD}, PknL_{CD}, and PknG, respectively. PknB from *M. tuberculosis*, one of the most studied mycobacterial STPK, possesses the two characteristic threonines in its activation loop (Thr-171 and Thr-173) (38), whereas two other *M. tuberculosis* kinases, PknE and PknH, only harbor a single Thr residue (49). Mass spectrometric profiling of the *C. glutamicum* STPKs phosphorylation sites indicated that PknA contains two phosphorylated Thr residues (Thr-179 and Thr-181) present in the activation loop. The corynebacterial PknB possesses one phosphorylated Thr (Thr-169) within its activation loop instead of two in its *M. tuberculosis* counterpart (Fig. 1 and Fig. 2). However, one cannot exclude the possibility that the Thr-171 was missed in our analysis. Together, these results suggest that the corynebacterial PknA and PknB enzymes present a classical mechanism of activation via the activation loop.

Surprisingly, unlike all the other kinases, PknL from *C. glutamicum* does not possess a classical TXT activation loop motif (Fig. 1). The TST motif found in *M. tuberculosis* PknL (31) is replaced by an SQD motif in *C. glutamicum* PknL. This was rather unexpected because PknL still undergoes autophosphorylation. These important differences may reflect mechanistic

differences of activation/regulation of PknL in the two microorganisms. Therefore, differential structure organizations (PknL possessing an extracytoplasmic region in *C. glutamicum*, which is not conserved in *M. tuberculosis*) may account for these mechanistic differences.

Concerning PknG, this kinase presents a completely different structural organization as being a cytoplasmic protein with no transmembrane region but still harboring a classical kinase domain. No phosphorylated residue could be identified within the activation loop. In fact, this region is missing in the PknG kinase, in both *C. glutamicum* and *M. tuberculosis* (Fig. 1), thus pointing to an unusual mechanism of phosphorylation of this kinase. In addition, mass spectrometric profiling of the PknG phosphorylation sites not only confirmed that this kinase is phosphorylated by the PknA, but also identified two unique phosphorylation sites, Thr-451 and Thr-787 located in the C terminus of the enzyme.

Thus, mass spectrometry profiling coupled to *in vitro* kinase assays unambiguously demonstrated the Ser/Thr kinase activity of the four *C. glutamicum* kinases. From a general standpoint, this type of analysis provides an essential groundwork for mechanistic/functional studies of bacterial STPKs and demonstrates the efficiency of combining genetics and mass spectrometry analyses with precise identification of phosphoacceptors, a prerequisite for a further understanding of the mode of action of PknA, PknB, PknL, and PknG. In particular, strains with defined mutations within the phosphorylation sites will be extremely helpful to establish the role of these kinases in corynebacterial growth and physiology. Consequently, this characterization significantly contributes to increasing our understanding of the biological mechanisms that control cellu-

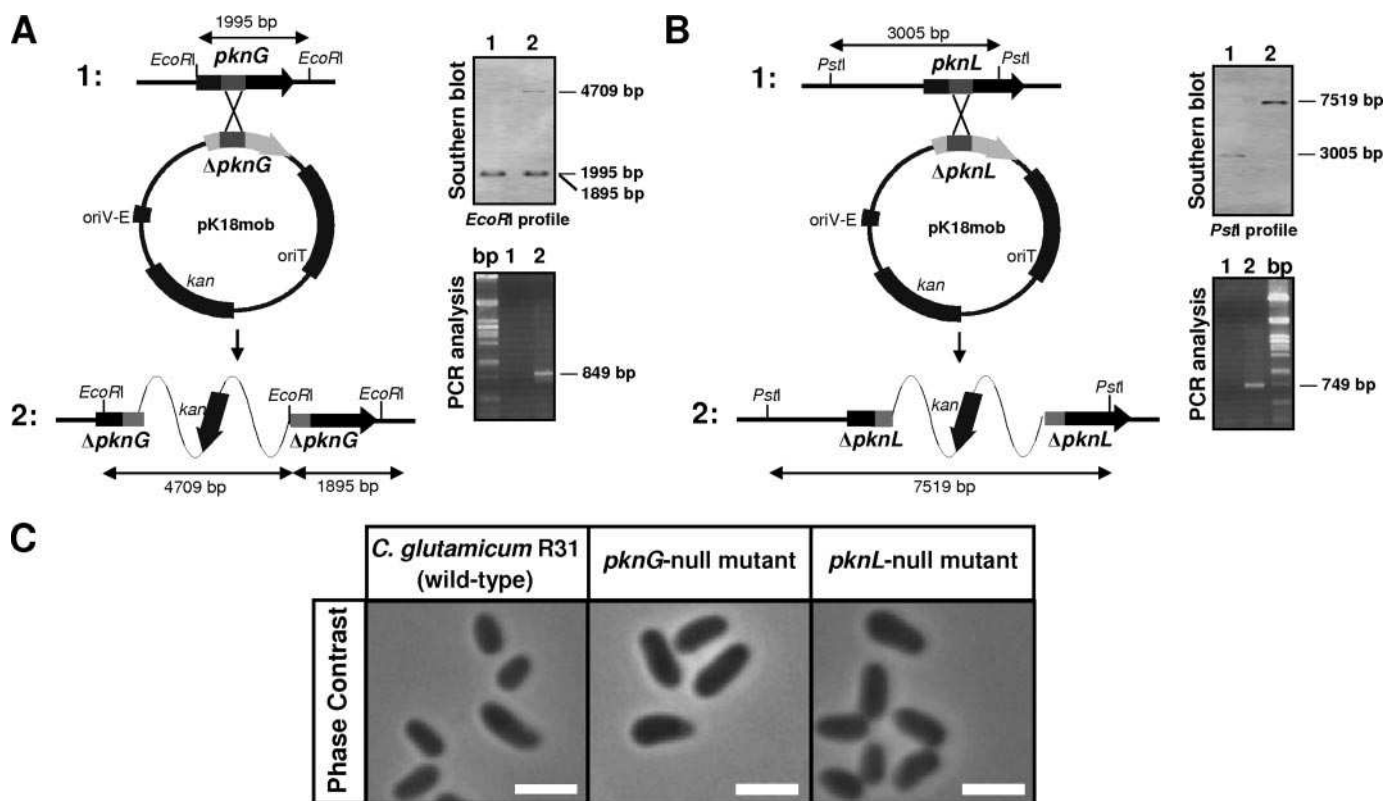


FIGURE 4. Construction of *C. glutamicum pknG* and *pknL* null mutants. *A*, schematic representation of the chromosomal region around *pknG* in *C. glutamicum* R31 (1) and in a *pknG* null mutant (2) obtained by integration of pK18mob into the chromosome of R31. Southern blot analysis of EcoRI-digested chromosomal DNA from R31 (lane 1) and *pknG* null mutant (lane 2) using an internal fragment of the *pknG* gene as probe. PCR analysis was performed using total chromosomal DNA from R31 (lane 1) and *pknG* null mutant (lane 2) using primers M13-26 and *pknLint2* (see Table 2). Size of the PCR fragments is represented in base pairs (bp). *B*, schematic representation of the chromosomal region around *pknL* in *C. glutamicum* R31 (1) and in a *pknL* null mutant (2) obtained by integration of pK18mob into the chromosome of R31. Southern blot analysis of PstI-digested chromosomal DNA from R31 (lane 1) and *pknL* null mutant (lane 2) using an internal fragment of the *pknL* gene as probe. PCR analysis was performed using total chromosomal DNA from R31 (lane 1) and *pknL* null mutant (lane 2) using primers M13-26 and *pknLint2* (see Table 2). Size of the PCR fragments is represented in base pairs (bp). *C*, microscopic images of *pknG* and *pknL* null mutants of *C. glutamicum*. Cells were observed in a Nikon E400 by phase contrast microscopy. The typical morphology of *C. glutamicum* R31 was conserved in strains deleted in *pknG* or *pknL*. Size bar, 1 μ m.

lar processes and cell regulation in this major industrial bacterial producer. In addition, characterization of these kinases should also help in examining their potential role in pathogenicity of the virulent species *Corynebacterium diphtheriae*, in which the four kinases are conserved (data not shown) (29).

Inactivation of *pknA*, *pknB*, *pknL*, and *pknG* in *C. glutamicum*—To further characterize the role of these kinases *in vivo*, and to appreciate the extent to which they are required for growth and viability of *C. glutamicum*, the corresponding chromosomal genes were inactivated using a previously described method (50). Niebisch *et al.* (15) demonstrated that a $\Delta pknG$ mutant was unable to grow on minimal medium with glutamine as sole carbon and nitrogen source. Therefore, *pknG* is not essential for *C. glutamicum* growth in complex medium (15). Gene disruption was carried out using internal fragments of *pknA*, *pknB*, and *pknL*. The internal fragment of *pknG* was used as control because *pknG* was not essential for the viability of *C. glutamicum* in complex medium. The conjugative suicide plasmids pKAint, pKBint, pKGint, and pKLint (Table 1) were introduced separately into *E. coli* S17-1 and mated with *C. glutamicum* R31 cells. *C. glutamicum* kanamycin-resistant transconjugants could only be obtained after mating with *E. coli* [pKGint] and *E. coli* [pKLint] (Fig. 4). These results suggest that inactivation of *pknA* or *pknB* leads to cell death; hence the

absence of *pknA* or *pknB* transconjugants is consistent with the conclusion that in *M. tuberculosis pknA* and *pknB* are essential genes (16, 51). However, the chromosomal *pknG* and *pknL* genes could be disrupted in the transconjugants *C. glutamicum* [pKGint] and *C. glutamicum* [pKLint]. Gene inactivation was confirmed by PCR analysis as well as by Southern blotting (Fig. 4, A and B). The *pknL* and *pknG* mutants showed a similar phenotype by phase contrast microscopy compared with the wild-type strain (Fig. 4C) and did not present any morphological alteration, suggesting that the *pknG* and *pknL* are not essential for viability at least in complex medium. Moreover, phenotype analysis of the corynebacterial strains overexpressing functional PknL or PknG did not reveal any differences compared with the wild-type control strain (data not shown).

Overall, our results suggest that the *C. glutamicum pknA* and *pknB* genes are essential, whereas the *pknL* and *pknG* genes are no longer necessary for growth. Thus, we focused our study on PknA and PknB, which appear to be key components of *C. glutamicum* growth and viability.

Partial Depletion of PknA or PknB Delays Growth and Produces Elongated Cells—Because PknA and PknB are essential, we were unable to examine the phenotypes of corynebacterial *pknA* or *pknB* null mutants. We therefore used a conditional expression system to reduce the amount of PknA or PknB in the

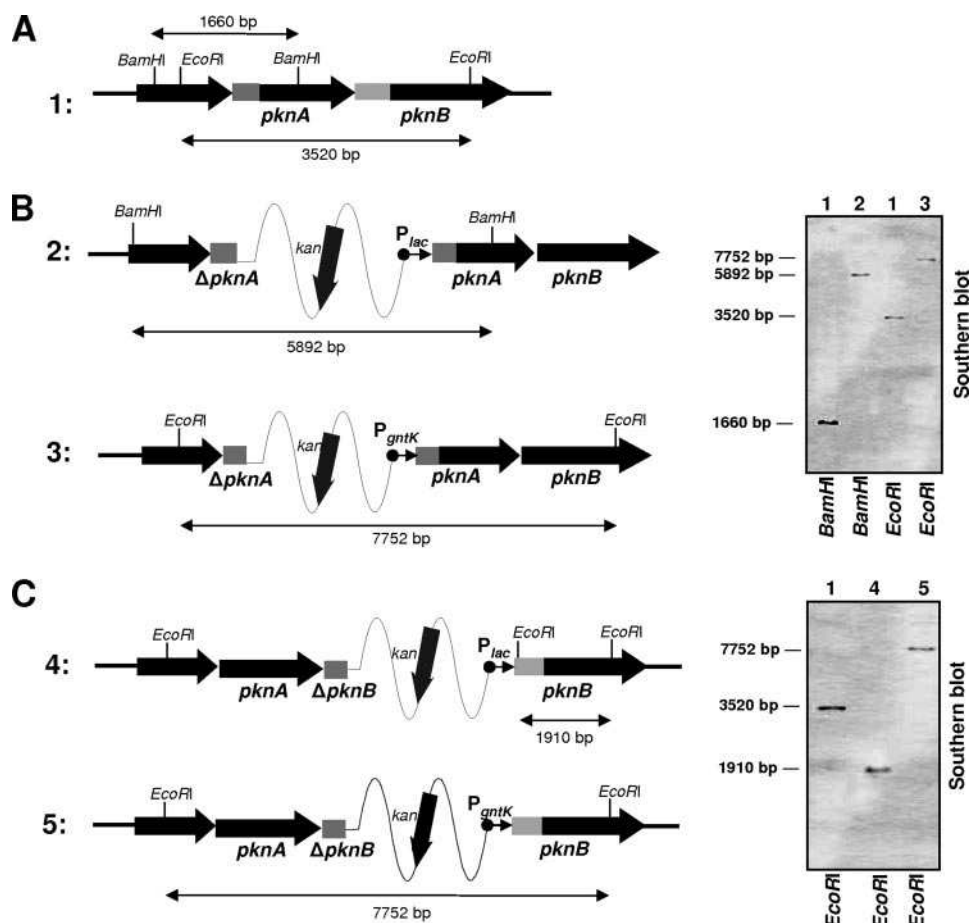


FIGURE 5. Construction of *C. glutamicum pknA* and *pknB* conditional expression strains. The suicide plasmids pKLA/B (P_{lac} - $\Delta pknA/B$) and pKPKA/B (P_{gntK} - $\Delta pknA/B$) were introduced into *C. glutamicum* by conjugation to disrupt the chromosomal copy of the gene and to introduce a functional copy of *pknA* or *pknB* under the control of the P_{lac} and P_{gntK} promoters, respectively. A, restriction map of the wild-type *pknA* and *pknB* locus (1). B, restriction maps of the P_{lac} - $\Delta pknA$ locus (2) and P_{gntK} - $\Delta pknA$ locus (3), respectively. Southern blot analysis of chromosomal DNA from P_{lac} - $\Delta pknA$ strain (lane 2) and P_{gntK} - $\Delta pknA$ strain (lane 3) digested with *Eco*RI or *Bam*HI. The probe used was an internal fragment of the *pknA* gene. C, restriction maps of the P_{lac} - $\Delta pknB$ locus (4) and P_{gntK} - $\Delta pknB$ locus (5), respectively. Southern blot analysis of chromosomal DNA from P_{lac} - $\Delta pknB$ strain (lane 4) and P_{gntK} - $\Delta pknB$ strain (lane 5) digested with *Eco*RI. The probe used was an internal fragment of the *pknB* gene.

cells to investigate their function. To this purpose, *C. glutamicum* was conjugated with *E. coli* carrying the suicide plasmids pKLA/B (P_{lac} - $\Delta pknA/B$) or pKPKA/B (P_{gntK} - $\Delta pknA/B$) (Table 1). These plasmids were designed to disrupt the chromosomal copy of the gene and to introduce a functional copy of the *pknA* or *pknB* gene under the control of the P_{lac} and P_{gntK} promoters, respectively. The P_{gntK} promoter is located upstream of the *gntK* gene encoding the *C. glutamicum* gluconate kinase and is regulated by sucrose (2). The *E. coli* P_{lac} promoter allows low expression levels in *C. glutamicum* (52). In *C. glutamicum*, pKLA/B and pKPKA/B were unable to replicate autonomously but integrated by homologous recombination into the chromosomal *pknA/B* locus, respectively. This resulted in the disruption of the targeted gene under its native promoter and the introduction of a functional copy of the corresponding gene placed under the control of the heterologous promoters P_{lac} or P_{gntK} (Fig. 5). Southern blot analysis (Fig. 5) and PCR analysis (data not shown) confirmed the correct pattern expected for integration of plasmids pKLA/B and pKPKA/B at the chromosomal *pknA* or *pknB* loci in the *C. glutamicum* transconjugant strains.

C. glutamicum strains under-expressing *pknA* or *pknB* from either the P_{lac} or P_{gntK} promoters showed an elongated phenotype that was undoubtedly different from the rod-shaped cells of the control strain *C. glutamicum* R31 (Fig. 6). These elongated bacteria were twice or three times the size of the wild-type bacteria and also appeared wider. In an attempt to determine whether these strains depleted in PknA or PknB were affected in cell wall biosynthesis, the cells were stained with Van-FL. Van-FL staining is often used to visualize the incorporation of newly synthesized PG at the sites of nascent PG synthesis during cell division in Gram-positive bacteria (4) because it is known that Van-FL is able to bind to the terminal D-Ala-D-Ala of the lipid-linked PG precursor (53). Fig. 6 indicates that in the *C. glutamicum* R31 control strain the newly synthesized PG was incorporated both at the cell poles and at the septum as reported earlier (4). In contrast, Van-FL staining of strains depleted in either PknA or PknB indicated that active PG biosynthesis was taking place at the cell poles as well as in multiple septa presumably corresponding to multiple division sites within the elongated *C. glutamicum* cells. This elongated phenotype could be the consequence of an error/defect in

the cell division process probably linked to the reduced level of PknA/B.

In addition, in an attempt to determine whether these strains depleted in PknA or PknB were affected in DNA segregation, cells were stained with 4'-6-diamidino-2-phenylindole (DAPI), which is able to form fluorescent complexes with natural double-stranded DNA, thus providing a useful tool in cytochemical investigations, and especially to visualize nucleoids (25). DAPI staining of the PknA- or PknB-depleted strains showed the presence of multiple nucleoids (Fig. 6), synonymous of several DNA replication events and suggesting that DNA segregation took place properly but that the final steps of cell division were inhibited. Similar phenotypes were reported in *C. glutamicum* with altered levels of FtsZ (52) and PBP2b (50). Interestingly, FtsZ and PBPA (homologous to *C. glutamicum* PBP2b) from *M. tuberculosis* are also substrates of PknA and PknB, respectively (17, 18).

We also investigated whether these major phenotype changes could affect *C. glutamicum* growth and viability. Cell growth of a PknA-depleted strain (Fig. 7A) as well as a PknB-depleted strain (Fig. 7B) was clearly delayed, characterized by

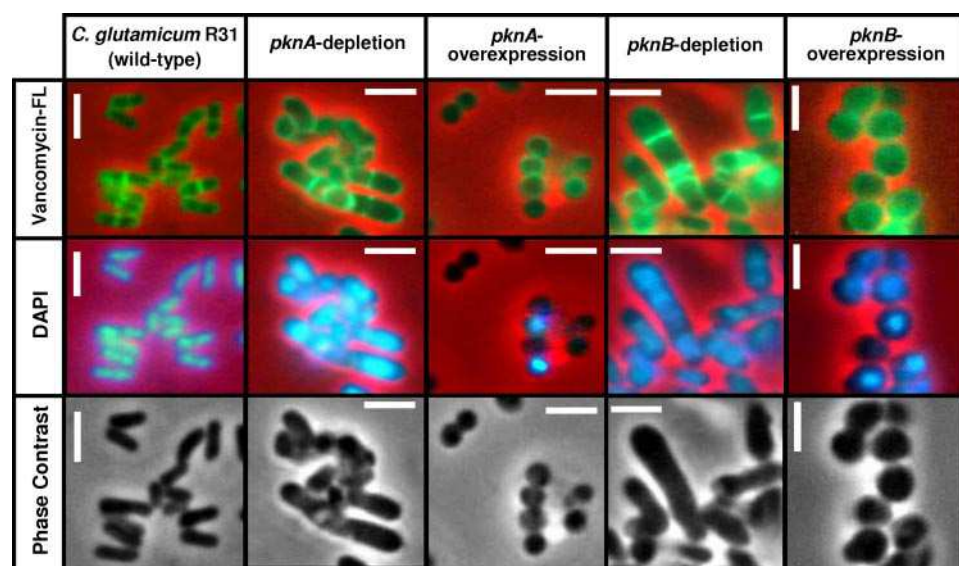


FIGURE 6. Microscopy of *C. glutamicum* strains with conditional expression of PknA and PknB levels. *C. glutamicum* cells were stained with vancomycin-FL or DAPI fluorescent dyes and observed using a Nikon E400 fluorescence microscope. The typical morphology of *C. glutamicum* R31 was converted to a coccoid morphology (no polar growth) in strains overexpressing *pknA* or *pknB*, whereas strain depleted for PknA or PknB grew apically and formed many apparently incomplete septa. Overlays of fluorescence images of vancomycin-FL (green) or DAPI (blue) are shown. Phase contrast images are also shown. Size bar, 1 μm .

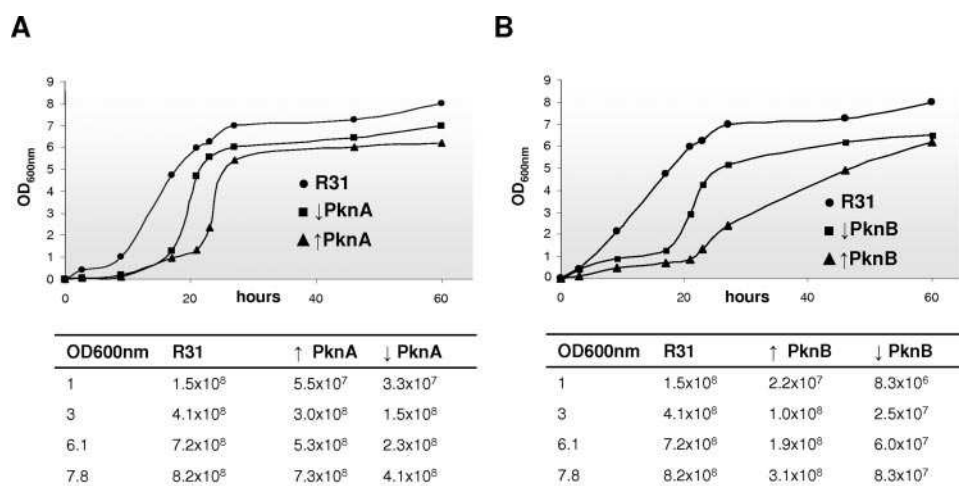


FIGURE 7. Growth and viability of *C. glutamicum* strains expressing different levels of the PknA and PknB kinases. A, strains with different levels of PknA: normal (R31), reduced (\downarrow), and increased (\uparrow). B, strains with different levels of PknB: normal (R31), reduced (\downarrow), and increased (\uparrow). Viable bacterial counts are expressed as the number of colony counts per ml. Numbers are representative of three independent experiments.

an important lag phase. In addition, these strains showed markedly diminished viability with a more severe effect caused by depleting PknB (about a 10-fold decrease compared with the wild-type R31 strain) than PknA (Fig. 7, A and B). Overall, these data suggest that low expression levels of PknA or PknB profoundly alter cell division. This interpretation is supported by the localization of *pknA* and *pknB* in a gene cluster that includes *rodA* and *pbp2b*, genes encoding orthologues of proteins that are important in this cell division and cell wall biosynthesis in corynebacteria (14).

Overexpression of PknA or PknB Delays Growth and Results in a Coccoid Cell-shaped Phenotype—We next examined the phenotypic effects of overexpressing PknA or PknB in *C. glutamicum*. Both the PknA- and the PknB-overexpressing strains were characterized by a delayed growth rate with a slightly

diminished viability than did the control R31 strain (Fig. 7, A and B), consistent with the results obtained in *M. tuberculosis* strains overexpressing the *M. tuberculosis* *pknA* or *pknB* genes (16). To observe the consequences of *pknA* or *pknB* overexpression on cell morphology, early exponential phase growing cells were harvested and examined microscopically. Interestingly, overexpression of either *pknA* or *pknB* leads to completely different morphotypes from those observed in the *pknA*- or *pknB*-depleted strains. Indeed, cells presented a characteristic coccoid-like phenotype compared with the control strain (Fig. 6). This particular cell shape suggests that cell elongation was strongly disturbed. In fact, Van-FL staining revealed incorporation of nascent PG around the cells indicating a lack of polar PG synthesis (Fig. 6). In addition, *C. glutamicum* cells overexpressing PknA or PknB form chains of cells attached at the poles, as seen for streptococci (54), suggesting a possible role of PknA and PknB in the final stages of cell division or cell-pole maturation.

In addition, DAPI staining highlights the fact that an important proportion of the cells overexpressing *pknA* or *pknB* was lacking detectable DNA, pointing to either a lack of chromosome segregation control (24) or to an indirect effect of the lack of polarization in the coccoid cells, which could be responsible for a DNA segregation problem (55, 56). This coccoid-like phenotype was rather unexpected, as it

was not observed in mycobacterial strains overexpressing the *M. tuberculosis* *pknA* or *pknB* genes (16). Although the effects on cell morphology are strongly supportive of a function of PknA and PknB kinases in the regulation of the cell shape and morphology in corynebacteria and mycobacteria, these results also suggest that the mechanisms, or the substrates, involved in these processes are different in the two organisms. For instance, Wag31, the *M. tuberculosis* homologue of the corynebacterial cell division protein DivIVA, a key protein that regulates growth, morphology, and polar cell wall synthesis, is phosphorylated by *M. tuberculosis* PknA and PknB (57). Thus, we have tested the ability of the *C. glutamicum* DivIVA protein to be a substrate of the four corynebacterial STPKs *in vitro* but failed to detect any phosphorylation (data not shown). Wag31 and DivIVA have two predicted coiled-coil regions that are inter-

rupted by a highly variable sequence (58). Interestingly, this highly variable sequence contains the unique phosphorylated site in mycobacterial Wag31 (Thr-73), which is not conserved in the *C. glutamicum* DivIVA protein (data not shown). This illustrates the fact that signal transduction pathways mediated by kinases for the regulation of cell shape in corynebacteria are likely to be different from mycobacteria. Our experimental data, along with previous work (16, 38, 47, 57, 59), strongly support a consistency of function of the PknB-like kinases in regulating cell shape and growth in a broad range of Gram-positive microorganisms.

In conclusion, our results provide, for the first time, a biochemical and comparative analysis of all four STPKs and the involvement of PknA and PknB in a signaling pathway in the process of cell shape control and cell division in corynebacteria. Our study also delineates important differences with the mycobacterial homologous kinases. In particular, we provided evidence that corynebacterial PknG, but not mycobacterial PknG, requires phosphorylation by PknA prior to transphosphorylation of its substrate OdhI. This suggests that, in addition to PknG, PknA may also participate in the phosphorylation status of OdhI and as a consequence, in the control of 2-oxoglutarate dehydrogenase, a key enzyme of the tricarboxylic acid cycle. A proteomic study revealed the presence of an important number of phosphorylated proteins in *C. glutamicum*, with the vast majority being metabolic enzymes rather than regulatory proteins, suggesting that protein phosphorylation plays a much broader function in the physiology of the bacteria than was previously expected (60). Therefore, further work needs to be carried out to understand how the limited number of kinases recognize an important number of substrates and how they participate in many complex signaling pathways. Another perspective of this work is the opening of a new field of investigation for future drug development to combat pathogenic corynebacterial species, such as *C. diphtheriae* or emerging corynebacterial pathogens. Because both PknA and PknB are essential, specific inhibitors capable of preventing corynebacterial growth would be extremely useful for the development of new therapies. In this context, recent studies demonstrated that mitoxantrone, a compound used in cancer treatment, represents a valid PknB inhibitor capable of preventing *M. tuberculosis* growth, suggesting that bacterial kinases represent a potential target for drug design (61). Whether mitoxantrone also inhibits corynebacterial growth remains to be established.

REFERENCES

- Hermann, T. (2003) *J. Biotechnol.* **104**, 155–172
- Letek, M., Valbuena, N., Ramos, A., Ordonez, E., Gil, J. A., and Mateos, L. M. (2006) *J. Bacteriol.* **188**, 409–423
- Letek, M., Ordonez, E., Fiuza, M., Honrubia-Marcos, P., Vaquera, J., Gil, J. A., Castro, D., and Mateos, L. M. (2007) *Int. Microbiol.* **10**, 271–282
- Daniel, R. A., and Errington, J. (2003) *Cell* **113**, 767–776
- Letek, M., Ordonez, E., Vaquera, J., Margolin, W., Flardh, K., Mateos, L. M., and Gil, J. A. (2008) *J. Bacteriol.* **190**, 3283–3292
- Scheffers, D. J., Jones, L. J., and Errington, J. (2004) *Mol. Microbiol.* **51**, 749–764
- Munoz-Dorado, J., Inouye, S., and Inouye, M. (1991) *Cell* **67**, 995–1006
- Zhang, W., Munoz-Dorado, J., Inouye, M., and Inouye, S. (1992) *J. Bacteriol.* **174**, 5450–5453
- Shi, L., Potts, M., and Kennelly, P. J. (1998) *FEMS Microbiol. Rev.* **22**, 229–253
- Grangeasse, C., Cozzzone, A. J., Deutscher, J., and Mijakovic, I. (2007) *Trends Biochem. Sci.* **32**, 86–94
- Stock, A. M., Robinson, V. L., and Goudreau, P. N. (2000) *Annu. Rev. Biochem.* **69**, 183–215
- Narayan, A., Sachdeva, P., Sharma, K., Saini, A. K., Tyagi, A. K., and Singh, Y. (2007) *Physiol. Genomics* **29**, 66–75
- Hett, E. C., and Rubin, E. J. (2008) *Microbiol. Mol. Biol. Rev.* **72**, 126–156
- Kalinowski, J., Bathe, B., Bartels, D., Bischoff, N., Bott, M., Burkovski, A., Dusch, N., Eggeling, L., Eikmanns, B. J., Gaigalat, L., Goesmann, A., Hartmann, M., Huthmacher, K., Kramer, R., Linke, B., McHardy, A. C., Meyer, F., Mockel, B., Pfefferle, W., Puhler, A., Rey, D. A., Ruckert, C., Rupp, O., Sahm, H., Wendisch, V. F., Wiegrabe, I., and Tauch, A. (2003) *J. Biotechnol.* **104**, 5–25
- Niebesch, A., Kabus, A., Schultz, C., Weil, B., and Bott, M. (2006) *J. Biol. Chem.* **281**, 12300–12307
- Kang, C. M., Abbott, D. W., Park, S. T., Dascher, C. C., Cantley, L. C., and Husson, R. N. (2005) *Genes Dev.* **19**, 1692–1704
- Dasgupta, A., Datta, P., Kundu, M., and Basu, J. (2006) *Microbiology* **152**, 493–504
- Thakur, M., and Chakraborti, P. K. (2006) *J. Biol. Chem.* **281**, 40107–40113
- Mateos, L. M., Schafer, A., Kalinowski, J., Martin, J. F., and Puhler, A. (1996) *J. Bacteriol.* **178**, 5768–5775
- Molle, V., Kremer, L., Girard-Blanc, C., Besra, G. S., Cozzzone, A. J., and Prost, J. F. (2003) *Biochemistry* **42**, 15300–15309
- Cohen-Gonsaud, M., Barthe, P., Pommier, F., Harris, R., Driscoll, P. C., Keep, N. H., and Roumestand, C. (2004) *J. Biomol. NMR* **30**, 373–374
- Molle, V., Brown, A. K., Besra, G. S., Cozzzone, A. J., and Kremer, L. (2006) *J. Biol. Chem.* **281**, 30094–30103
- Kieser, T., Bibb, M. J., Buttner, M. J., Chen, B. F., and Hopwood, D. A. (2000) *Practical Streptomyces Genetics*, pp. 161–220, John Innes Institute, Norwich, UK
- Letek, M., Fiuza, M., Ordonez, E., Villadaneos, A. F., Ranos, A., Mateos, L. M., and Gil, J. A. (2008) *Antonie Leeuwenhoek* 10.1007/S10482-008-9224-4
- Daniel, R. A., Harry, E. J., and Errington, J. (2000) *Mol. Microbiol.* **35**, 299–311
- Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem.* **68**, 850–858
- Leonard, C. J., Aravind, L., and Koonin, E. V. (1998) *Genome Res.* **8**, 1038–1047
- Nishio, Y., Nakamura, Y., Kawarabayasi, Y., Usuda, Y., Kimura, E., Sugimoto, S., Matsui, K., Yamagishi, A., Kikuchi, H., Ikeo, K., and Gojobori, T. (2003) *Genome Res.* **13**, 1572–1579
- Cerdeno-Tarraga, A. M., Efstratiou, A., Dover, L. G., Holden, M. T., Pallen, M., Bentley, S. D., Besra, G. S., Churcher, C., James, K. D., De Zoysa, A., Chillingworth, T., Cronin, A., Dowd, L., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Moule, S., Quail, M. A., Rabinowitsch, E., Rutherford, K. M., Thomson, N. R., Unwin, L., Whitehead, S., Barrell, B. G., and Parkhill, J. (2003) *Nucleic Acids Res.* **31**, 6516–6523
- Tauch, A., Kaiser, O., Hain, T., Goesmann, A., Weisshaar, B., Albersmeier, A., Bekel, T., Bischoff, N., Brune, I., Chakraborty, T., Kalinowski, J., Meyer, F., Rupp, O., Schneiker, S., Viehoveer, P., and Puhler, A. (2005) *J. Bacteriol.* **187**, 4671–4682
- Canova, M. J., Veyron-Churlet, R., Zanella-Cleon, I., Cohen-Gonsaud, M., Cozzzone, A. J., Becchi, M., Kremer, L., and Molle, V. (2008) *Proteomics* **8**, 521–533
- Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., III, Tekaija, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) *Nature* **393**, 537–544
- Bentley, S. D., Chater, K. F., Cerdeno-Tarraga, A. M., Challis, G. L., Thomson, N. R., James, K. D., Harris, D. E., Quail, M. A., Kieser, H., Harper, D.,

Serine/Threonine Protein Kinases from *C. glutamicum*

- Bateman, A., Brown, S., Chandra, G., Chen, C. W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C. H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Rabbinowitsch, E., Rajandream, M. A., Rutherford, K., Rutter, S., Seeger, K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B. G., Parkhill, J., and Hopwood, D. A. (2002) *Nature* **417**, 141–147
34. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) *Science* **241**, 42–52
35. Hanks, S. K., and Hunter, T. (1995) *FASEB J.* **9**, 576–596
36. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132
37. Yeats, C., Finn, R. D., and Bateman, A. (2002) *Trends Biochem. Sci.* **27**, 438–440
38. Boitel, B., Ortiz-Lombardia, M., Duran, R., Pompeo, F., Cole, S. T., Cervenansky, C., and Alzari, P. M. (2003) *Mol. Microbiol.* **49**, 1493–1508
39. Peirs, P., De Wit, L., Braibant, M., Huygen, K., and Content, J. (1997) *Eur. J. Biochem.* **244**, 604–612
40. Molle, V., Girard-Blanc, C., Kremer, L., Doublet, P., Cozzone, A. J., and Prost, J. F. (2003) *Biochem. Biophys. Res. Commun.* **308**, 820–825
41. Zhang, W., Inouye, M., and Inouye, S. (1996) *Mol. Microbiol.* **20**, 435–447
42. Hanlon, W. A., Inouye, M., and Inouye, S. (1997) *Mol. Microbiol.* **23**, 459–471
43. Nariya, H., and Inouye, S. (2005) *Mol. Microbiol.* **58**, 367–379
44. Shirai, T., Nakato, A., Izutani, N., Nagahisa, K., Shioya, S., Kimura, E., Kawarabayashi, Y., Yamagishi, A., Gojobori, T., and Shimizu, H. (2005) *Metab. Eng.* **7**, 59–69
45. Cowley, S., Ko, M., Pick, N., Chow, R., Downing, K. J., Gordhan, B. G., Betts, J. C., Mizrahi, V., Smith, D. A., Stokes, R. W., and Av-Gay, Y. (2004) *Mol. Microbiol.* **52**, 1691–1702
46. Koul, A., Choidas, A., Tyagi, A. K., Drlica, K., Singh, Y., and Ullrich, A. (2001) *Microbiology* **147**, 2307–2314
47. Villarino, A., Duran, R., Wehenkel, A., Fernandez, P., England, P., Brodin, P., Cole, S. T., Zimny-Arndt, U., Jungblut, P. R., Cervenansky, C., and Alzari, P. M. (2005) *J. Mol. Biol.* **350**, 953–963
48. Molle, V., Zanella-Cleon, I., Robin, J. P., Mallejac, S., Cozzone, A. J., and Becchi, M. (2006) *Proteomics* **6**, 3754–3766
49. Av-Gay, Y., and Everett, M. (2000) *Trends Microbiol.* **8**, 238–244
50. Valbuena, N., Letek, M., Ordonez, E., Ayala, J., Daniel, R. A., Gil, J. A., and Mateos, L. M. (2007) *Mol. Microbiol.* **66**, 643–657
51. Fernandez, P., Saint-Joanis, B., Barilone, N., Jackson, M., Gicquel, B., Cole, S. T., and Alzari, P. M. (2006) *J. Bacteriol.* **188**, 7778–7784
52. Ramos, A., Letek, M., Campelo, A. B., Vaquera, J., Mateos, L. M., and Gil, J. A. (2005) *Microbiology* **151**, 2563–2572
53. Sheldrick, G. M., Jones, P. G., Kennard, O., Williams, D. H., and Smith, G. A. (1978) *Nature* **271**, 223–225
54. Fadda, D., Santona, A., D'Ulisse, V., Ghelardini, P., Ennas, M. G., Whalen, M. B., and Massidda, O. (2007) *J. Bacteriol.* **189**, 1288–1298
55. Young, K. D. (2006) *Microbiol. Mol. Biol. Rev.* **70**, 660–703
56. Karczmarek, A., Martinez-Arteaga, R., Alexeeva, S., Hansen, F. G., Vicente, M., Nanninga, N., and den Blaauwen, T. (2007) *Mol. Microbiol.* **65**, 51–63
57. Kang, C. M., Nyayapathy, S., Lee, J. Y., Suh, J. W., and Husson, R. N. (2008) *Microbiology* **154**, 725–735
58. Flardh, K. (2003) *Mol. Microbiol.* **49**, 1523–1536
59. Duran, R., Villarino, A., Bellinzoni, M., Wehenkel, A., Fernandez, P., Boitel, B., Cole, S. T., Alzari, P. M., and Cervenansky, C. (2005) *Biochem. Biophys. Res. Commun.* **333**, 858–867
60. Bendt, A. K., Burkovski, A., Schaffer, S., Bott, M., Farwick, M., and Hermann, T. (2003) *Proteomics* **3**, 1637–1646
61. Wehenkel, A., Fernandez, P., Bellinzoni, M., Catherinot, V., Barilone, N., Labesse, G., Jackson, M., and Alzari, P. M. (2006) *FEBS Lett.* **580**, 3018–3022
62. Schafer, A., Kalinowski, J., Simon, R., Seep-Feldhaus, A. H., and Puhler, A. (1990) *J. Bacteriol.* **172**, 1663–1666
63. Santamaria, R. I., Gil, J. A., and Martin, J. F. (1985) *J. Bacteriol.* **162**, 463–467
64. Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G., and Puhler, A. (1994) *Gene (Amst.)* **145**, 69–73
65. Jager, W., Schafer, A., Puhler, A., Labes, G., and Wohlleben, W. (1992) *J. Bacteriol.* **174**, 5462–5465

The MurC Ligase Essential for Peptidoglycan Biosynthesis Is Regulated by the Serine/Threonine Protein Kinase PknA in *Corynebacterium glutamicum**

Received for publication, September 16, 2008, and in revised form, October 28, 2008. Published, JBC Papers in Press, October 29, 2008, DOI 10.1074/jbc.M807175200

Maria Fiuza^{†1}, Marc J. Canova[§], Delphine Patin[¶], Michal Letek[‡], Isabelle Zanella-Cléon[§], Michel Becchi[§],
Luís M. Mateos[‡], Dominique Mengin-Lecreux^{¶||}, Virginie Molle^{§2,3}, and José A. Gil^{†3}

From the [†]Departamento de Biología Molecular, Área de Microbiología, Facultad de Biología, Universidad de León, León 24071, Spain, the [§]Institut de Biologie et Chimie des Protéines, UMR 5086, CNRS, Université Lyon1, IFR128 BioSciences, Lyon-Gerland, 7 passage du Vercors, Lyon 69367, Cedex 07, France, the [¶]University Paris-Sud, Institut de Biochimie et Biophysique Moléculaire et Cellulaire, UMR 8619, Orsay F-91405, France, and the ^{||}CNRS, Laboratoire des Enveloppes Bactériennes et Antibiotiques, UMR 8619, Orsay F-91405, France

The Mur ligases play an essential role in the biosynthesis of bacterial cell-wall peptidoglycan and thus represent attractive targets for the design of novel antibacterials. These enzymes catalyze the stepwise formation of the peptide moiety of the peptidoglycan disaccharide peptide monomer unit. MurC is responsible of the addition of the first residue (L-alanine) onto the nucleotide precursor UDP-MurNAc. Phosphorylation of proteins by Ser/Thr protein kinases has recently emerged as a major physiological mechanism of regulation in prokaryotes. Herein, the hypothesis of a phosphorylation-dependent mechanism of regulation of the MurC activity was investigated in *Corynebacterium glutamicum*. We showed that MurC was phosphorylated *in vitro* by the PknA protein kinase. An analysis of the phosphoamino acid content indicated that phosphorylation exclusively occurred on threonine residues. Six phosphoacceptor residues were identified by mass spectrometry analysis, and we confirmed that mutagenesis to alanine residues totally abolished PknA-dependent phosphorylation of MurC. *In vitro* and *in vivo* ligase activity assays showed that the catalytic activity of MurC was impaired following mutation of these threonine residues. Further *in vitro* assays revealed that the activity of the MurC-phosphorylated isoform was severely decreased compared with the non-phosphorylated protein. To our knowledge, this is the first demonstration of a MurC ligase phosphorylation *in vitro*. The finding that phosphorylation is correlated with a decrease in MurC enzymatic activity could have significant consequences in the regulation of peptidoglycan biosynthesis.

Due to the increasing number of antibiotic-resistant strains and the emergence of new pathogenic microorganisms, one of

the biggest challenges for modern biomedical research is the continuous development of new antimicrobial drugs targeting bacterial essential mechanisms such as cell division or peptidoglycan (PG)⁴ biosynthesis (1). The bacterial cell wall PG is a giant molecule that sustains the shape of the bacterial cell and contains the outward forces generated in maintaining an osmotic pressure gradient against the environment. Without this PG layer the cell integrity would be ruptured, and this could lead to cell death. Therefore the PG biosynthesis machinery represents a promising source of putative targets for antibacterial chemotherapy (2, 3).

The biosynthesis of bacterial PG is a complex two-stage process (4). The first stage involves the assembly of the disaccharide peptide monomer unit by enzymes located in the cytoplasm or at the inner surface of the cytoplasmic membrane (3, 5). The peptide moiety of the monomer unit is assembled stepwise by the successive additions of L-alanine, D-glutamic acid, meso-diaminopimelic acid or L-lysine, and D-alanyl-D-alanine to UDP-N-acetylmuramic acid (UDP-MurNAc). These steps are catalyzed by specific peptide synthetases (ligases), which are designated as MurC, MurD, MurE, and MurF, respectively, all participating in non-ribosomal peptide bond formation with the concomitant hydrolysis of ATP. The MurNAc-pentapeptide motif of the resulting nucleotide precursor is then transferred by the MraY translocase onto the undecaprenyl phosphate carrier molecule, generating the lipid intermediate I. The subsequent addition of the N-acetylglucosamine motif of UDP-GlcNAc onto lipid I generates lipid II in a reaction catalyzed by MurG (6). The second stage of the PG biosynthesis consists in the polymerization by transglycosylation and transpeptidation reactions of the disaccharide pentapeptide monomers, a reaction taking place in the periplasmic space and that is catalyzed by the penicillin-binding proteins.

The PG biosynthesis pathway enzymes, which are essential and specific for bacteria, represent important potential targets for screening novel antibacterial compounds. Due to the growing emergence of bacterial multiresistance to currently used antibiotics, the discovery of new therapeutic compounds has

* This work was supported in part by grants from the Région Rhône-Alpes (to M. C.); by the CNRS, the University of Lyon (France), and the National Research Agency (ANR-06-MIME-027-01 to V. M.); by the Junta de Castilla y León (Ref. LE040A07 to J. A. G.); by the Ministerio de Ciencia y Tecnología (Spain) (Grants BIO2008-00519 and BIO2005-02723 to J. A. G. and L. M. M.); and by the CNRS and Université Paris-Sud XI (Grant UMR 8619 to D. M. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[†] A beneficiary of a fellowship from the Ministerio de Educación y Ciencia.

² To whom correspondence may be addressed. Tel.: 33-4-72-72-26-79; Fax: 33-4-72-72-26-41; E-mail: vmolle@ibcp.fr.

³ To whom correspondence may be addressed. Tel.: 34-987-29-15-03; Fax: 34-987-29-14-09; E-mail: jagils@unileon.es.

⁴ The abbreviations used are: PG, peptidoglycan; MurNAc, N-acetylmuramic acid; STPK, Ser/Thr protein kinase; GST, glutathione S-transferase; MS, mass spectrometry; MS/MS, tandem MS; LC, liquid chromatography; ESI, electrospray ionization; TEV, tobacco etch virus.

MurC Phosphorylation in *C. glutamicum*

indeed become a necessity. In recent years, an extensive search for specific inhibitors interfering with the cytoplasmic steps of this pathway, and in particular with the four steps catalyzed by the Mur ligases, has been developed (2, 7, 8). The UDP-MurNAc:L-alanine ligase (MurC), encoded by the *murC* gene, represents such an interesting candidate for drug development (9, 10). Recently, different phosphinic acid derivatives and substrate analogues have been identified as Mur ligase inhibitors (11, 12).

Corynebacterium glutamicum is a rod-shaped non-pathogenic Gram-positive actinomycete widely used in the industrial production of amino acids such as L-lysine and L-glutamic acid (13). *C. glutamicum* has been extensively studied as a model microorganism due to the strategies employed by this actinomycete to achieve a rod-shaped morphology. In fact, the mechanisms taking place in *C. glutamicum* happened to be completely different from that of *Escherichia coli* or *Bacillus subtilis* (14, 15), whereas the number of genes involved in cell division and PG biosynthesis in *C. glutamicum* is lower (16).

Interestingly, an earlier work on the phosphoproteome of *C. glutamicum* (17) identified MurC as being phosphorylated *in vivo*, suggesting that protein phosphorylation plays a much broader function in *C. glutamicum* than was previously expected. Recently, we described the characterization of the four STPKs from *C. glutamicum* ATCC 13869 and highlighted their role in cell division (18). Moreover, Thakur and Chakraborti (19) showed that MurD from *Mycobacterium tuberculosis* was phosphorylated by the Ser/Thr protein kinase (STPK) PknA, although no further characterization of the role of the phosphorylation on the MurD enzyme activity was investigated. Therefore, it was tempting to speculate that MurC in *C. glutamicum* could also be regulated by STPK phosphorylation.

The focus of this work is to study the regulation of MurC in *C. glutamicum* via phosphorylation. As a first step in deciphering the potential role/participation of the corynebacterial STPKs in the regulation of MurC activity, we confirmed its specific phosphorylation by the PknA kinase through a combination of *in vitro* phosphorylation assays and mass spectrometric identification of the different MurC phosphorylation sites. Moreover, we demonstrated that the murein ligase activity of MurC was negatively regulated upon its phosphorylation. To our knowledge, this work represents the first evidence of a Mur enzyme regulated by phosphorylation.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Bacterial strains and plasmids are described in Table 1. Strains used for cloning and expression of recombinant proteins were *E. coli* TOP10 (Invitrogen) and *E. coli* BL21(DE3)Star (Stratagene), respectively. *E. coli* cells were grown and maintained at 37 °C in LB medium supplemented with 100 µg/ml ampicillin and/or 50 µg/ml kanamycin, when required. The *murC* temperature-sensitive *E. coli* strain H1119 was grown at 30 °C in 2YT (1.6% Bactotrypton, 1.0% Bactoyeast extract, 0.5% NaCl, pH 7.0) medium and was used for genetic complementation experiments with plasmids carrying wild-type or mutated copies of the *murC* gene. *C. glutamicum* cells were grown at 30 °C in TSB (Trypticase soy broth, Oxoid) or TSA (TSB containing 2% agar)

medium supplemented with 12.5 µg/ml kanamycin. Plasmids to be transferred by conjugation from *E. coli* to corynebacteria were introduced by transformation into the donor strain *E. coli* S17-1. Mobilization of plasmids from *E. coli* S17-1 to *C. glutamicum* R31 was accomplished as described previously (20).

Cloning, Expression, and Purification of MurC Proteins—First, the *murC* gene was cloned to generate a recombinant MurC protein expressed in *E. coli*. Therefore, the *murC* gene was amplified by PCR using *C. glutamicum* ATCC 13869 genomic DNA as a template and the primers pair murC1/murC2 (Table 2), containing NdeI and NheI restriction sites, respectively. The 1461-bp amplified product was digested by NdeI and NheI and ligated to the pETTev vector (Table 1) generating the pTEVmurC plasmid. *E. coli* BL21(DE3)Star cells transformed with this construction were used for expression and purification of His₆-tagged MurC, as previously described (21). Finally, the purified His₆-tagged MurC was treated with TEV protease according to the manufacturer's instructions (Invitrogen). Secondly, overexpression and purification of MurC from *C. glutamicum* cultures was performed using standard PCR strategies. The *murC* gene from *C. glutamicum* was amplified using the primers pair murC1/murHisNdeI2 (Table 2). The PCR product carrying a His tag at its C-terminal end was digested with NdeI and subsequently cloned under the control of the *Pdiv* promoter into plasmid pEDiv (Table 1). The resulting expression vector, named pEDivmurHis, was introduced by conjugation into *C. glutamicum* R31. Purification of the soluble His₆-tagged MurC protein from *C. glutamicum* was performed as described previously (21).

In Vitro Kinase Assays—*In vitro* phosphorylation was performed with 2 µg of MurC in 20 µl of buffer P (25 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol, 5 mM MgCl₂, 1 mM EDTA) with 200 µCi/ml [γ -³³P]ATP corresponding to 65 nM (PerkinElmer Life Sciences, 3000 Ci/mmol), and 0.5 µg of kinase. Plasmids pGEXA, pGEXB, pGEXL, and pTEVGfull (Table 1) were used for the expression and purification in *E. coli* of the four recombinant STPKs from *C. glutamicum* as previously described (18). After 15-min incubation, the reaction was stopped by adding sample buffer and heating the mixture at 100 °C for 5 min. The reaction mixtures were analyzed by SDS-PAGE. After electrophoresis, gels were soaked in 20% trichloroacetic acid for 10 min at 90 °C, stained with Coomassie Blue, and dried. Radioactive proteins were visualized by autoradiography using direct exposure films.

Analysis of the Phosphoamino Acid Content of Proteins—MurC sample (5 µg) phosphorylated *in vitro* by the GST-tagged PknA, and unreacted [γ -³³P]ATP were separated by one-dimensional gel electrophoresis and electroblotted onto an Immobilon polyvinylidene difluoride membrane. The ³³P-labeled protein bands were detected by autoradiography and excised from the Immobilon blot and hydrolyzed in 6 M HCl for 1 h at 110 °C. The acid-stable phosphoamino acids released were separated by electrophoresis in the first dimension at pH 1.9 (800 V/h) in 7.8% acetic acid, 2.5% formic acid, followed by ascending chromatography in the second dimension in 2-methyl-1-propanol/formic acid/water (8:3:4, v/v). After migration, radioactive compounds were detected by autoradiography. Authentic phosphoserine, phosphothreonine and

TABLE 1
Bacterial strains and plasmids used in this study

Strains or plasmids	Genotype or description	Source or reference
<i>E. coli</i> TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>endA1</i> <i>nupG</i> ; used for general cloning	Invitrogen
<i>E. coli</i> BL21(DE3)Star	F2 <i>ompT</i> <i>hsdSB</i> (rB2 mB2) <i>gal dcm</i> (DE3); used to express recombinant proteins in <i>E. coli</i>	Stratagene
<i>E. coli</i> H1119	<i>murC</i> temperature-sensitive mutant	(23)
<i>E. coli</i> S17-1	Mobilizing donor strain, <i>pro recA</i> , which possesses an RP4 derivative integrated into the chromosome	(38)
<i>C. glutamicum</i> 13869	Wild-type control strain	ATCC
<i>C. glutamicum</i> R31	<i>C. glutamicum</i> ATCC 13869; derivative used as recipient in conjugation experiments	(39)
pETTev	pET15b (Novagen) derivative including the replacement of the thrombin site coding sequence with a tobacco etch virus (TEV) protease site	(40)
pTEV <i>murC</i>	pTEV derivative used to express His-tagged fusion of MurC	This work
pGEX4T-3	<i>E. coli</i> vector designed to make GST gene fusions	GE Healthcare
pGEXA	pGEX4T-3 derivative used to express GST fusion of PknA cytoplasmic domain	(18)
pGEXB	pGEX4T-3 derivative used to express GST fusion of PknB cytoplasmic domain	(18)
pGEXL	pGEX4T-3 derivative used to express GST fusion of PknL cytoplasmic domain	(18)
pTEVGfull	pETTev derivative used to express His-tagged PknG	(18)
pEDiv	Mobilizable plasmid able to replicate in <i>E. coli</i> and <i>C. glutamicum</i> ; <i>kan</i> and <i>cat</i> resistance genes	A. Ramos (unpublished)
pEDiv <i>murHis</i>	pEDiv derivative used to express His-tagged fusion of MurC in <i>C. glutamicum</i>	This work
pGEX <i>pknAT179A</i>	pGEXA derivative used to express GST fusion of PknA cytoplasmic domain carrying the mutation T179A	This work
pGEX <i>pknAT181A</i>	pGEXA derivative used to express GST fusion of PknA cytoplasmic domain carrying the mutation T181A	This work
pGEX <i>pknAT179/181A</i>	pGEXA derivative used to express GST fusion of PknA cytoplasmic domain carrying the mutations T179A and T181A	This work
pGEX <i>pknAK49A</i>	pGEXA derivative used to express GST fusion of PknA cytoplasmic domain carrying the mutation K49A	This work
pTEV <i>murC1T</i>	pTEV <i>murC</i> derivative used to express His-tagged fusion of MurC1T carrying the mutation T362A	This work
pTEV <i>murC2T</i>	pTEV <i>murC1T</i> derivative used to express His-tagged fusion of MurC2T carrying the mutation T362A/T365A	This work
pTEV <i>murC3T</i>	pTEV <i>murC2T</i> derivative used to express His-tagged fusion of MurC3T carrying the mutation T362A/T365A/T51A	This work
pTEV <i>murC4T</i>	pTEV <i>murC3T</i> derivative used to express His-tagged fusion of MurC4T carrying the mutation T362A/T365A/T51A/T120A	This work
pTEV <i>murC5T</i>	pTEV <i>murC4T</i> derivative used to express His-tagged fusion of MurC5T carrying the mutation T362A/T365A/T51A/T120A/T167A	This work
pTEV <i>murC6T</i>	pTEV <i>murC5T</i> derivative used to express His-tagged fusion of MurC6T carrying the mutation T362A/T365A/T51A/T120A/T167A/T133A	This work
pTEV <i>murCT51A</i>	pTEV <i>murC</i> derivative used to express His-tagged fusion of MurCT51A carrying the mutation T51A	This work
pTEV <i>murCT120A</i>	pTEV <i>murC</i> derivative used to express His-tagged fusion of MurCT120A carrying the mutation T120A	This work
pTEV <i>murCT133A</i>	pTEV <i>murC</i> derivative used to express His-tagged fusion of MurCT133A carrying the mutation T133A	This work
pTEV <i>murCT167A</i>	pTEV <i>murC</i> derivative used to express His-tagged fusion of MurCT167A carrying the mutation T167A	This work
pTEV <i>murCT362A</i>	pTEV <i>murC</i> derivative used to express His-tagged fusion of MurCT362A carrying the mutation T362A	This work
pTEV <i>murCT365A</i>	pTEV <i>murC</i> derivative used to express His-tagged fusion of MurCT365A carrying the mutation T365A	This work
pTrc99A	<i>E. coli</i> vector allowing high level expression under the IPTG inducible <i>trc</i> promoter	Pharmacia
pTrc99 <i>murC</i>	pTrc99A derivative carrying <i>murC</i>	This work
pTrc99 <i>murC1T</i>	pTrc99A derivative carrying <i>murC1T</i> (T362A)	This work
pTrc99 <i>murC2T</i>	pTrc99A derivative carrying <i>murC2T</i> (T362A/T365A)	This work
pTrc99 <i>murC3T</i>	pTrc99A derivative carrying <i>murC3T</i> (T362A/T365A/T51A)	This work
pTrc99 <i>murC4T</i>	pTrc99A derivative carrying <i>murC4T</i> (T362A/T365A/T51A/T120A)	This work
pTrc99 <i>murC5T</i>	pTrc99A derivative carrying <i>murC5T</i> (T362A/T365A/T51A/T120A/T167A)	This work
pTrc99 <i>murC6T</i>	pTrc99A derivative carrying <i>murC6T</i> (T362A/T365A/T51A/T120A/T167A/T133A)	This work

phosphotyrosine were run in parallel and visualized by staining with ninhydrin.

Cloning and Purification of PknA Mutant Proteins—Site-directed mutagenesis was directly performed on the pGEXA expression plasmid (Table 1) using inverse-PCR amplification with the following self-complementary primers (Table 2): N-pknAT179A/C-pknAT179A, N-pknAT181A/C-pknAT181A, N-pknAT179A-T181A/C-pknAT179A-T181A, and N-pknAK49M/C-pknAK49M to generate pGEX*pknAT179A*, pGEX*pknAT181A*, pGEX*pkn-*

AT179A/T181A, and pGEX*pknAK49M*, respectively (Table 1). All constructs were verified by DNA sequencing. The different GST-tagged recombinant fusion proteins were overexpressed and purified as reported earlier (18).

Site-directed Mutagenesis—The six threonine residues from *C. glutamicum* MurC identified by mass spectrometry after *in vitro* phosphorylation with GST-tagged PknA were replaced by alanine residues by site-directed mutagenesis using inverse-PCR amplification. A first PCR was carried out using pTEV*murC* (Table 1) as a

MurC Phosphorylation in *C. glutamicum*

TABLE 2
Primers used in this study

Primer	Gene	5' to 3' Sequence ^{a,b}
murC1	<i>murC</i>	GGAATTCATATGGTGACCACTCCACAC (NdeI)
murC2	<i>murC</i>	TGGAATTCGCTAGCCTAATTGTTTTGCAGCTGATCC (NheI)
murHisNdeI	<i>murC</i>	GGAATTCATATGCTAATGATGATGATGATGATGATTGTTTTGCAGC (NdeI)
N-pknAK49M	<i>pknA</i>	GATCGCGAAGTAGCCATC ATGG TACTGCGCCCGAATTTCC
C-pknAK49M	<i>pknA</i>	GGAAAATTCGGGGCGCAGTAC CCAT GATGGCTACTTCGCGATC
N-pknAT179A	<i>pknA</i>	GCCGCTGCTGTGCCTTTG GCC CGCACCCGCATGGTGGTG
C-pknAT179A	<i>pknA</i>	CACCACCATGCCGGTGC GGC CAAAGGCACAGCAGCGGC
N-pknAT181A	<i>pknA</i>	GCTGTGCCTTTGACCCGC GCC GGCATGGTGGTGGTACT
C-pknAT181A	<i>pknA</i>	AGTACCCACCACCATGCC GCC CGGGTCAAAGGCACAGC
N-pknAT179A/T181A	<i>pknA</i>	GCCGCTGCTGTGCCTTTG GCC CGCG GCC GGCATGGTGGTGGGT
C-pknAT179A/T181A	<i>pknA</i>	ACCCACCACCATGCC GCC GGCG GCC CAAAGGCACAGCAGCGGC
N-murC362	<i>murC</i>	GATTACGCACACCACC GCG GAAGTAACTGCAGTGCTC
C-murC362	<i>murC</i>	GAGCACTGCAGTTACTT CGC TGGGTGGTGTGCGTAATC
N-murC362/365	<i>murC</i>	GCACACCACCC GCG GAAGT AGCT GCAGTGCTCAGCGCTGCG
C-murC362/365	<i>murC</i>	GCCAGCGCTGAGCACTGC AGC TACTTCC CGC TGGGTGGTGTGC
N-murC365	<i>murC</i>	CACCACCCAACGGAAGT AGCT GCAGTGCTCAGCGCGGGC
C-murC365	<i>murC</i>	CGCAGCGCTGAGCACTGC AGC TACTTCCGTTGGGTGGTG
N-murC51	<i>murC</i>	GATGCGAAAGATTCCCGC GCC TTGCTTCCACTCCGCGGC
C-murC51	<i>murC</i>	GGCGCGGAGTGAAGCA AGG CGCGGAATCTTTGGCATC
N-murC120	<i>murC</i>	GAATTGCTGGAAGGCTCC GCC CAGGCTCTTGATCGCGGGT
C-murC120	<i>murC</i>	ACCCGCGATCAAGACCT GCG CGGAGCCTTCCAGCAATTC
N-murC167	<i>murC</i>	ACCAATGCGCACCATGG AGCT GGTGGTCTTTATCGCT
C-murC167	<i>murC</i>	AGCGATAAAGACCTCACC AGC TCCATGGTGGCATTGGT
N-murC133	<i>murC</i>	ACCCACGGTAAGACCTCC GCC ACCCTATGTCTGTGGTA
C-murC133	<i>murC</i>	TACCACAGACATAGAGGT GCC GAGGCTTACCCTGGGT
murH1119-1	<i>murC</i>	TTTAATCATGACCACCTCCACTTGG (BspHI)
murH1119-2	<i>murC</i>	CTTACAGATCTTAATTGTTTTGCAGCTG (BglII)

^a Restriction sites are underlined and specified by brackets.

^b Mutagenized codons are shown in bold.

template with the primers pair N-murC362 and C-murC362 (Table 2) to generate pTEV*murC*1T (T362A). A second PCR was carried out using pTEV*murC* (Table 1) as a template with the primers pair N-murC362/365 and C-murC362/365 (Table 2) to generate pTEV*murC*2T (T362A/T365A). This mutant and the subsequent additional mutants were used as templates in subsequent PCR reactions using the following primers pairs: N-murC51 and C-murC51, N-murC120 and C-murC120, N-murC167 and C-murC167, and N-murC133 and C-murC133 (see Table 2) to generate pTEV*murC*3T (T362A/T365A/T51A), pTEV*murC*4T (T362A/T365A/T51A/T120A), pTEV*murC*5T (T362A/T365A/T51A/T120A/T167A), and pTEV*murC*6T (T362A/T365A/T51A/T120A/T167A/T133A), respectively. Individual mutants were generated using pTEV*murC* (Table 1) as a template with the primers pairs N-murC51/C-murC51, N-murC120/C-murC120, N-murC133/C-murC133, N-murC167/C-murC167, N-murC362/C-murC362, and N-murC365/C-murC365 (Table 2) to generate pTEV*murC*5T1A, pTEV*murC*120A, pTEV*murC*133A, pTEV*murC*167A, pTEV*murC*362A, and pTEV*murC* T365A, respectively (Table 1). All the resulting constructs were verified by DNA sequencing. The different His₆-tagged mutant proteins were overexpressed and purified, as described above.

MS Analysis—Purified wild-type and mutant MurC proteins were subjected to *in vitro* phosphorylation by GST-tagged PknA as described above, excepted that [γ -³³P]ATP was replaced with 5 mM ATP. Subsequent analyses using NanoLC/nanospray/tandem mass spectrometry (LC-ESI/MS/MS) were performed as previously described (18).

Immunoblotting—Corynebacterial MurC purified either from *E. coli* or *C. glutamicum* was loaded on a 10% polyacrylamide gel, electrophoresed, blotted on polyvinylidene difluoride, and detected using either monoclonal mouse anti-phospho-threonine, -serine, -tyrosine, or polyclonal rabbit anti-His

antibodies used at 1:100 and 1:10,000 dilution, respectively. Alkaline phosphatase-conjugated anti-mouse or anti-rabbit was used as a secondary antibody at a 1:5,000 dilution.

Complementation with *C. glutamicum* MurC—Plasmids pTEV*murC*, pTEV*murC*1T, pTEV*murC*2T, pTEV*murC*3T, pTEV*murC*4T, pTEV*murC*5T, and pTEV*murC*6T were used as templates for PCR amplification of the *murC* gene with the primers pair murH1119-1/murH1119-2 (Table 2) containing a BspHI and BglII restriction site, respectively. The resulting 1461-bp products, carrying the wild-type and mutated gene copies, respectively, were digested with BspHI and BglII, and cloned into the plasmid vector pTrc99A (22) between the compatible NcoI and BamHI sites, generating pTrc*murC*, pTrc*murC*1T, pTrc*murC*2T, pTrc*murC*3T, pTrc*murC*4T, pTrc*murC*5T, and pTrc*murC*6T, respectively. These plasmids allowing high level expression of the *C. glutamicum* MurC and MurC mutants under the control of the strong isopropyl 1-thio- β -D-galactopyranoside-inducible *trc* promoter, were transformed in the *E. coli* MurC temperature-sensitive mutant strain H1119 (23). Transformants were grown at the permissive temperature (30 °C) before being shifted to the restrictive temperature (42 °C). Complementation was judged by the ability of the mutant to grow at the restrictive temperature.

UDP-MurNAc L-alanine Ligase Assay—The L-alanine-adding activity of wild-type and mutant MurC proteins was assayed according to Liger *et al.* (24) by following the formation of UDP-MurNAc-L-[¹⁴C]alanine in 40 μ l of reaction mixture containing 100 mM Tris-HCl, pH 8.6, 20 mM MgCl₂, 20 mM ammonium sulfate, 0.5 mM L-[¹⁴C]alanine (0.6 KBq, 5.5 Gbq/mmol, Amersham), 1 mM UDP-MurNAc, 5 mM ATP, and enzyme (25 μ l of an appropriate dilution in buffer A: 20 mM potassium phosphate, pH 7.2, containing 1 mM dithiothreitol and 10% glycerol). The mixture was incubated at 37 °C for 30 min and the reaction was stopped by addition of 8 μ l of acetic acid,

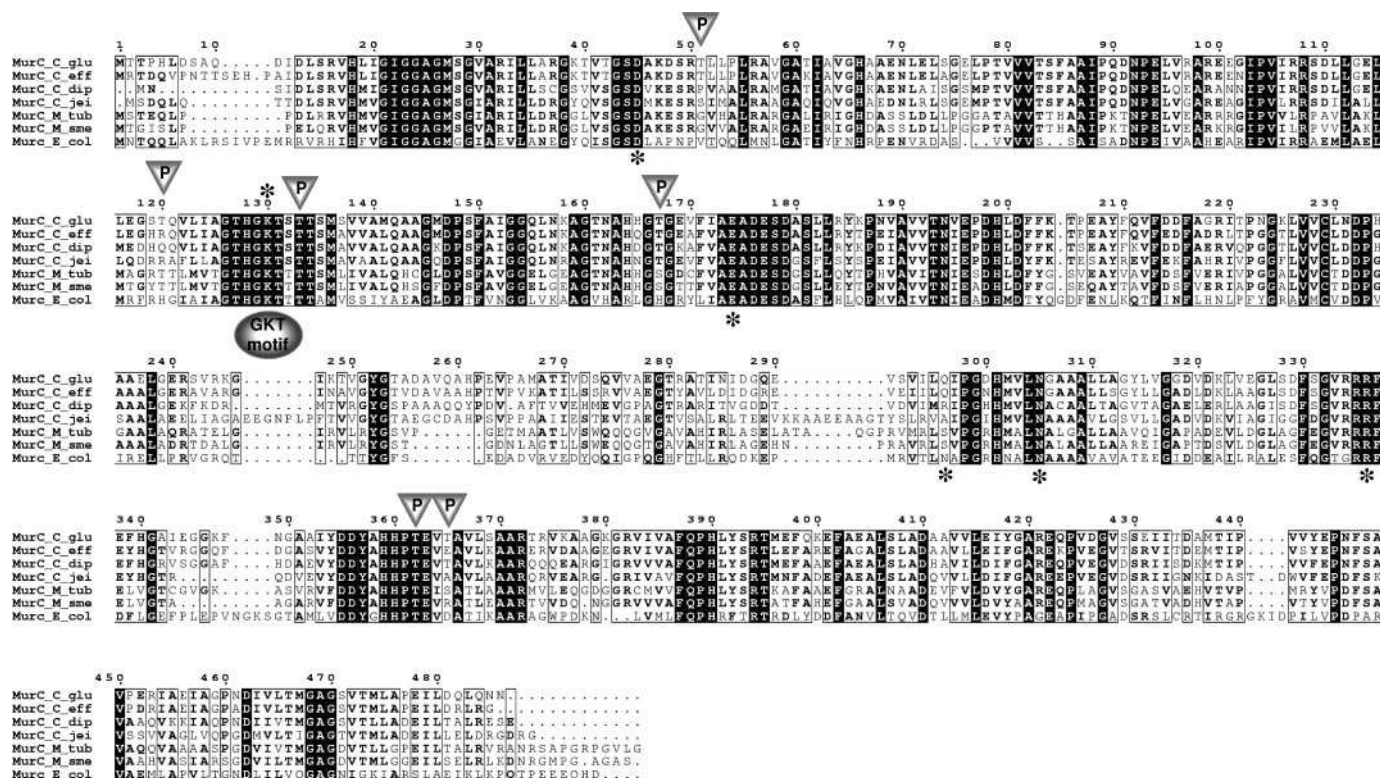


FIGURE 1. Multiple sequence alignment of MurC ortholog proteins from corynebacteria, mycobacteria, and *E. coli*. The alignment was performed using ClustalW and Esript programs (*C. glu*, *C. glutamicum*; *C. eff*, *C. efficiens*; *C. dip*, *C. diphtheriae*; *C. jei*, *C. jeikeium*; *M. tub*, *M. tuberculosis*; *M. sme*, *M. smegmatis*; and *E. coli*, *E. coli*). The conserved GKT motif involved in ATP binding is represented by a shaded oval. Triangles represent PknA-dependent phosphorylation sites (Thr⁵¹, Thr¹²⁰, Thr¹³³, Thr¹⁶⁷, Thr³⁶², and Thr³⁶⁵) of *C. glutamicum* MurC. Residues indicated by an asterisk in the *E. coli* MurC sequence correspond to residues that were previously identified as invariant within the whole Mur ligase family (3, 25): the Asp⁵⁰, Lys¹³⁰ (GKT motif), and Glu¹⁷⁴ residues involved in the catalytic process, and the Asn²⁹⁶ and Arg³²⁷ residues involved in the structural organization of the active site. Numbering of amino acids corresponds to the MurC protein from *C. glutamicum*.

followed by lyophilization. To test the effect of the phosphorylation of MurC on its enzyme activity, the assay was performed as described above excepted that the MurC enzyme (20 μ g/ml) was preincubated overnight at 37 °C with or without wild-type PknA (16 μ g/ml), or with PknA_K49M mutant (30 μ g/ml) in buffer A supplemented with 2 mM ATP and 5 mM MgCl₂. The radioactive product UDP-MurNAc-L-[¹⁴C]alanine and substrate L-[¹⁴C]alanine were separated by reversed-phase high-performance liquid chromatography on a Nucleosil 5C18 column (4.6 \times 150 mm, Alltech France) using 50 mM ammonium formate, pH 3.9, as the eluent, at a flow rate of 0.6 ml/min. Detection was performed with a radioactive flow detector (model LB506-C1, Berthold France, La Garenne-Colombes, France) using the Quicksafe Flow 2 scintillator (Zinsser Analytic, Maidenhead, UK) at 0.6 ml/min. Quantitation was carried out with the Radiostar software (Berthold).

RESULTS AND DISCUSSION

MurC Is a Substrate of the *C. glutamicum* PknA—The peptide moiety of the PG monomer unit is assembled stepwise in the cytoplasm by the successive actions of four Mur ligases designated as MurC, MurD, MurE, and MurF. These enzymes share limited sequence identity but have several highly conserved regions that map primarily to the active site. Each of them comprises three structural domains: an N-terminal domain with a Rossmann-type fold primarily responsible for binding of the UDP-MurNAc-(peptide) substrate, a large cen-

tral ATP-binding (ATPase) domain, and a C-terminal domain associated with binding of the amino acid substrate (25). Based on protein sequence alignments and on the three-dimensional structure of the *E. coli* MurC protein (26), these three domains in the *C. glutamicum* MurC may extend between Met¹–Gly¹¹⁸, Ser¹¹⁹–Arg³³⁴, and Arg³³⁵–Asn⁴⁸⁶, respectively (Figs. 1 and 4). By comparing the amino acid sequences of the MurC, -D, -E, and -F ligases from different bacterial genera, several conserved residues were identified. This analysis led to the characterization of the ATP-binding motif located in the conserved sequence (GXXGK(T/S)), and therefore named the GKT motif (Fig. 1). There are also a number of common conserved amino acid residues present in the different Mur ligases. Taking *E. coli* as a model, it was established that Asp⁵⁰, Lys¹³⁰, Glu¹⁷⁴, and Asp³⁵¹ were essential for the catalytic process of these ligases, whereas residues His¹⁹⁹, Asn²⁹³, Asn²⁹⁶, and Arg³²⁷ were involved in the structure of the active site. The sequence of several MurC proteins from representative species of mycobacteria and corynebacteria were aligned using the ClustalW and Esript programs (Fig. 1). The invariant residues Asp⁵⁰, Lys¹³⁰, Glu¹⁷⁴, Asn²⁹⁶, and Arg³²⁷ appeared conserved in all the sequences aligned, thus confirming that MurC from *C. glutamicum* harbors most of the characteristics of a functional Mur ligase enzyme. Therefore, the recombinant MurC protein was expressed and purified from *E. coli* BL21(DE3)Star harboring the pTEV_{MurC}. The protein contained an N-terminal His

MurC Phosphorylation in *C. glutamicum*

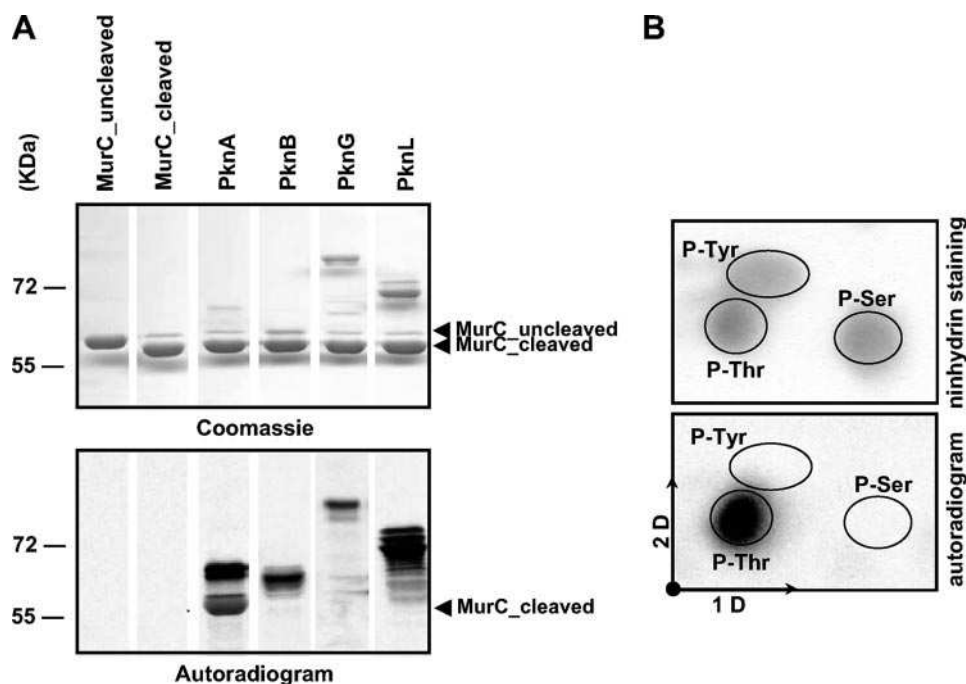


FIGURE 2. *In vitro* phosphorylation of *C. glutamicum* MurC. **A**, *in vitro* phosphorylation of MurC by corynebacterial STPKs. The four recombinant STPKs (PknA, PknB, PknG, and PknL) were expressed and purified as previously described by Fiuza *et al.* (18). Recombinant MurC was treated with the TEV protease to remove the N-terminal His tag and then incubated with [γ - 33 P]ATP and the different kinases. Samples were separated by SDS-PAGE (upper panel) and visualized by autoradiography (lower panel). Upper bands illustrate the autokinase activity of each STPK, whereas lower bands reflect phosphorylation of MurC. **B**, phosphoamino acid content of MurC. MurC was phosphorylated *in vitro* in presence of PknA and [γ - 33 P]ATP, analyzed by SDS-PAGE, electroblotted onto an Immobilon polyvinylidene difluoride membrane, excised, and hydrolyzed in acid. The phosphoamino acids thus liberated were separated by electrophoresis in the first dimension (1D) and ascending chromatography in the second dimension (2D). After migration, radioactive molecules were detected by autoradiography (lower panel). Authentic phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) were run in parallel as internal standard controls, and visualized by ninhydrin staining (upper panel).

tag, which was subsequently removed after cleavage with the TEV protease. Analysis of the purified recombinant protein by SDS-PAGE revealed that the protein was expressed in a soluble form migrating slightly above its predicted molecular mass of 52 kDa (Fig. 2A).

Previous work on the phosphoproteome of *C. glutamicum* (17) identified MurC as being phosphorylated *in vivo*. Moreover, we have recently reported the characterization of the four STPKs from *C. glutamicum* ATCC 13869 and highlighted their role in cell division (18). These results prompted us to investigate whether MurC would also represent a substrate for corynebacterial STPKs. A systematic approach was used to investigate whether STPKs of *C. glutamicum* (PknA, PknB, PknG, or PknL) could phosphorylate MurC. All these STPKs were expressed and purified from *E. coli* as described previously (18). The different STPKs migrate as diffuse bands reflecting the different levels of phosphorylation for each isoform, and this aberrant profile of migration of STPKs kinases has already been reported in earlier studies (27–29). Interestingly, when STPKs were incubated in the presence of recombinant MurC and [γ - 33 P]ATP, phosphorylation of MurC was specifically observed with the PknA kinase, whereas PknB and PknL, which display autophosphorylation activity *in vitro*, did not phosphorylate MurC (Fig. 2A). The PknG kinase needs to be activated by the PknA kinase to trigger its autophosphorylation activity, therefore PknG was phosphorylated by PknA as previously

described and then used to transphosphorylate MurC (18). As shown on Fig. 2A, MurC did not show any radioactive band, thus indicating that PknG, even if activated by its cognate kinase PknA, could not phosphorylate MurC *in vitro*. Moreover, MurC alone is unable to incorporate [γ - 33 P]ATP, confirming that MurC is a substrate of PknA (Fig. 2A).

We next determined the nature of the amino acid residues phosphorylated in MurC by analyzing the phosphoamino acid content of PknA-phosphorylated MurC. The MurC protein (5 μ g) was labeled with [γ - 33 P]ATP *in vitro*, separated by SDS-PAGE, excised, and subjected to acid hydrolysis. Fig. 2B shows that MurC was exclusively phosphorylated on threonine residues.

Together, these data suggest a specificity of substrate recognition by PknA toward MurC. Although a specific interaction between MurC and PknA may exist, it remains to be established whether this specific partnering also occurs *in vivo*.

The Activation Loop Thr¹⁷⁹ and Thr¹⁸¹ Residues Are Essential for the

Autophosphorylation Activity of *C. glutamicum* PknA—Diverse mechanisms of eukaryotic protein kinase regulation have been described. The transition between active and inactive forms may occur via control of access to the catalytic site and/or the substrate-binding site. These regulatory mechanisms involve phosphorylation/dephosphorylation via an autocatalytic mechanism or the action of other kinases and phosphatases. The activation loop appears to be a major control element of an active/inactive conformational switch in numerous kinases (30), and the conformation of the activation loop often depends on the phosphorylation state (31). Based on structural studies, it is thought that the activation loop controls both the accessibility to the catalytic site and the binding of the substrate. Recently, Canova *et al.* (27) demonstrated that the phosphorylated residues Thr¹⁷³ and Thr¹⁷⁵ present in the activation loop were essential for the autophosphorylation activity of the *M. tuberculosis* PknL kinase, and that phosphorylation of the Thr¹⁷³ residue was also required for optimal PknL-mediated phosphorylation of its substrate Rv2175c. Interestingly, the corresponding threonine residues Thr¹⁷⁹ and Thr¹⁸¹ found in the *C. glutamicum* PknA were recently identified as phosphorylation sites (18). The presence of such residues in PknA from *C. glutamicum* supports the concept that phosphorylation of the activation loop could play a regulatory role, as recently described for other mycobacterial STPKs (27, 32, 33).

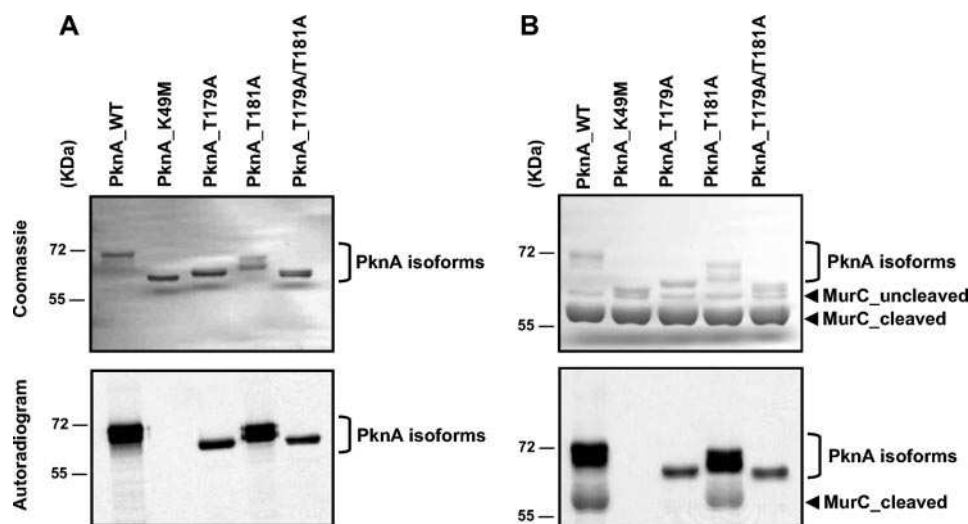


FIGURE 3. *In vitro* phosphorylation of MurC by PknA and the PknA activation loop mutants. *A*, *in vitro* phosphorylation of the different PknA activation loop mutants. All proteins were overproduced in *E. coli* and purified as GST fusions. The following proteins were incubated with [γ - 33 P]ATP: PknA_WT, PknA_K49M, PknA_T179A, PknA_T181A, and PknA_T179A/T181A. Proteins were separated by SDS-PAGE and stained with Coomassie Blue (*upper panel*), and the radioactive bands were revealed by autoradiography (*lower panel*). *B*, *in vitro* phosphorylation of MurC by PknA and the different PknA activation loop mutants. Recombinant MurC was treated with the TEV protease to remove the N-terminal His tag and then used in phosphorylation assays in the presence of [γ - 33 P]ATP and PknA_WT, PknA_K49M, PknA_T179A, PknA_T181A, or PknA_T179A/T181A. Proteins were separated by SDS-PAGE and stained with Coomassie Blue (*upper panel*), and the radioactive bands were revealed by autoradiography (*lower panel*).

To determine a possible link between Thr¹⁷⁹ and Thr¹⁸¹ and the autophosphorylation activity of PknA, the two residues were individually substituted by Ala to generate PknA_T179A and PknA_T181A, respectively. In addition, a double mutant was also created and designated PknA_T179A/T181A. These PknA mutants were expressed and purified from *E. coli* as previously described (18). The electrophoretic migration profile showed that the different PknA mutants migrated differently from the wild-type protein (Fig. 3A, *upper panel*). We reasoned that, because these proteins displayed different electrophoretic mobility properties, they must differ in their intrinsic phosphorylation states. This hypothesis was confirmed by labeling the mutant proteins *in vitro* with [γ - 33 P]ATP. PknA_T179A gave rise to a lower radioactive signal than the wild-type enzyme (Fig. 3A, *lower panel*). Quantification of the signal intensity indicated an autophosphorylation activity of 47% with respect to the activity of the wild-type protein, which was arbitrarily placed at 100%. This result argues that Thr¹⁷⁹ is a key phosphorylation site of the activation loop, necessary for PknA autophosphorylation activity. The signal intensity generated by PknA_T181A was intermediate between those of the wild-type and Thr¹⁷⁹ mutant proteins (Fig. 3A, *lower panel*), representing 81% of residual activity with respect to the wild-type protein activity. Therefore, this result confirms the requirement of Thr¹⁸¹ for optimal PknA autophosphorylation activity, although this residue appears less important than Thr¹⁷⁹. When both Thr residues were mutated, the intensity of the signal was similar to the one of the single Thr¹⁷⁹ mutant (Fig. 3A, *lower panel*). Together, these results confirm that double phosphorylation of the activation loop residues Thr¹⁷⁹ and Thr¹⁸¹ is necessary for full kinase activity and unambiguously demonstrate the role of both phosphothreonines.

To exclude the possibility of exogenous contamination that could explain labeling of PknA with [γ - 33 P]ATP, Lys⁴⁹ was mutated to Met. Purified PknA_K49M, was incubated in the presence of [γ - 33 P]ATP, and as expected, no signal could be detected as Lys⁴⁹ is essential for catalyzing the phosphorylation reaction, in agreement with previous reports (Fig. 3A, *lower panel*) (34, 35). This confirms that the radioactive signals observed for the different isoforms of the PknA kinase are only representing the autophosphorylation activity of PknA and not a contamination from the purification procedure.

Phosphorylation of MurC Is Dependent on the PknA Activation Loop Thr¹⁷⁹ Residue—Several recent publications indicated that phosphorylation of the threonine residues present in the STPK activation loop is not only necessary for controlling the kinase activity but is

also required for recruitment and phosphorylation of its substrate (27, 36). These results prompted us to analyze the contribution of the activation loop key residues of PknA (Thr¹⁷⁹ and Thr¹⁸¹) in the transphosphorylation mechanism between the kinase and its substrate MurC. This was achieved by incubating recombinant MurC with PknA_K49M, PknA_T179A, PknA_T181A, or PknA_T179A/T181A in the presence of [γ - 33 P]ATP (Fig. 3B). PknA_K49M was unable to phosphorylate *C. glutamicum* MurC, indicating that phosphorylation of PknA is a prerequisite to the transphosphorylation reaction (Fig. 3B, *lower panel*). More importantly, whereas the T179A mutation completely abolished the transphosphorylation reaction, replacement of Thr¹⁸¹ by Ala did not alter PknA-dependent phosphorylation of MurC (Fig. 3B, *lower panel*). Furthermore, MurC could not be phosphorylated by PknA_T179A/T181A, clearly demonstrating that phosphorylation of MurC is dependent on Thr¹⁷⁹. The finding that MurC interacts only with the phosphorylated form of PknA and that this interaction is abolished by the T179A substitution suggests that this phosphopeptide recognition motif is involved in protein-protein interaction between the two corynebacterial partners, as previously described in its related actinomycete *M. tuberculosis* (27).

MurC Is Phosphorylated on Multiple Threonine Residues—The role of post-translational mechanisms like phosphorylation in regulatory processes or the effect of phosphorylation on a given substrate in the physiology and/or cell division represent key events to our understanding of the signaling mechanisms through serine/threonine phosphorylation. This usually requires the identification of the phosphorylated sites, which often remains very challenging. To identify which of the 31 threonine residues of the *C. glutamicum* MurC corresponded to the phosphorylated site(s), a mass spectrometry approach

MurC Phosphorylation in *C. glutamicum*

TABLE 3

Phosphorylation status of recombinant MurC as determined by mass spectrometry

Phosphorylated tryptic and chymotryptic peptide sequence	Number of detected phosphate groups	Phosphorylated residue(s)
	LC-ESI/MS/MS	
[348–372] FNAAITDDYAHHPTEVTAVLSAAR	1	Thr ³⁶²
[348–372] FNAAITDDYAHHPTEVTAVLSAAR	1	Thr ³⁶⁵
[348–372] FNAAITDDYAHHPTEVTAVLSAAR	2	Thr ³⁶² and Thr ³⁶⁵
[40–56] TVTGSDAKDSRTLLPLR	1	Thr ⁵¹
[48–56] DSRTLLPLR	1	Thr ⁵¹
[108–130] RSDLLGELLEGSTQVLIAGTHGK	1	Thr ¹²⁰
[109–130] SDLLGELLEGSTQVLIAGTHGK	1	Thr ¹²⁰
[159–184] AGTNAHHGTGEVFIAEADSDASLLR	1	Thr ¹⁶⁷
[131–158] TSTTSMVVMQAAGMDPSFAIGGQLNK	1	Thr ¹³³
[124–141] IAGTHGKTSTTSMVVM	1	Thr ¹³³

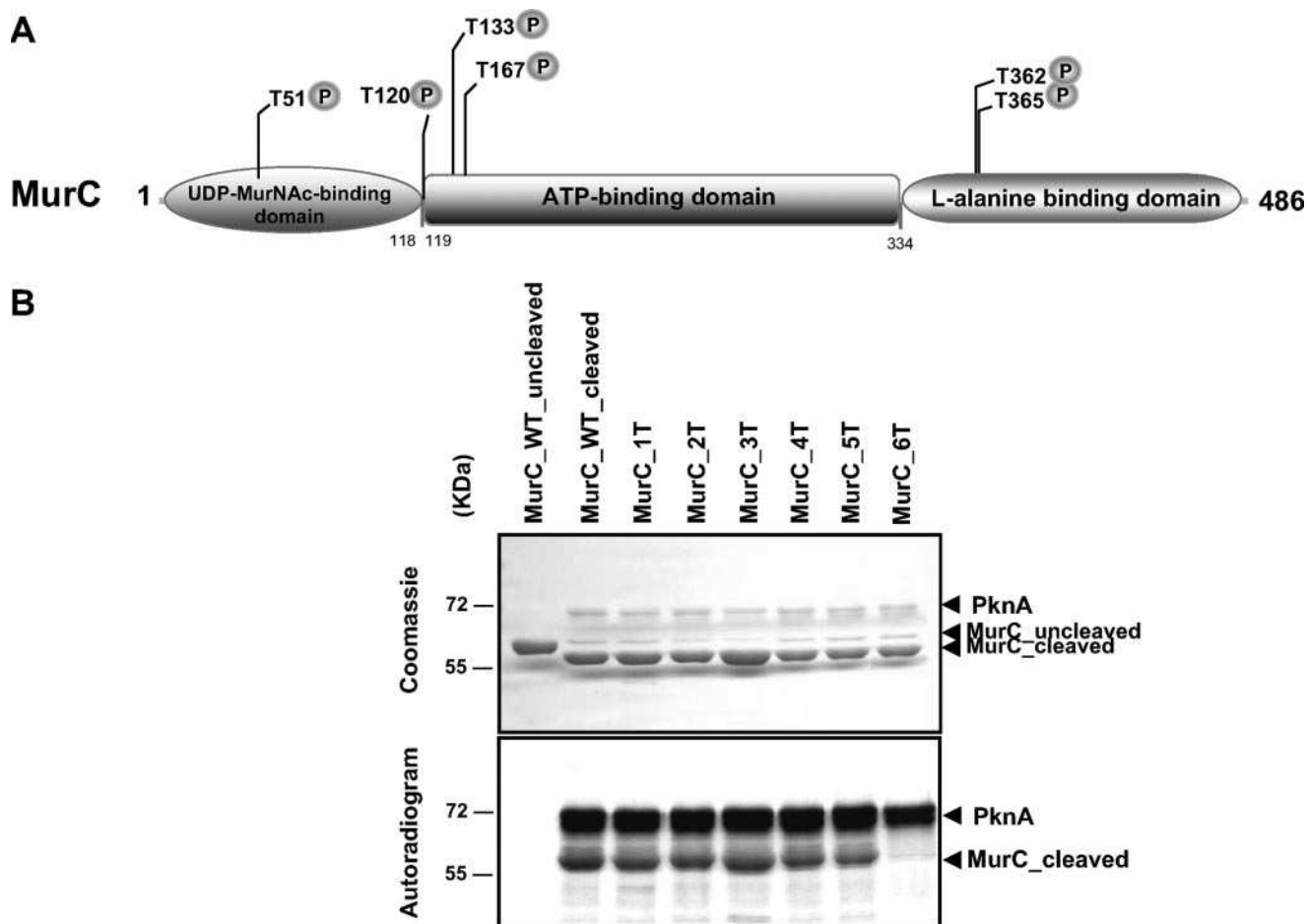


FIGURE 4. Structural organization of MurC and phosphorylation of MurC mutants by PknA. *A*, schematic representation of MurC from *C. glutamicum*. The MurC protein comprises three structural domains, an N-terminal domain responsible for the binding of the UDP-MurNAc substrate, a central ATP-binding (ATPase) domain, and a C-terminal domain involved in the binding of the amino acid substrate L-alanine. These domains are shown by shaded circles and boxes. The phosphorylation sites identified in MurC are indicated by a "P." *B*, *in vitro* phosphorylation of MurC mutants by PknA. The different MurC mutants were treated with the TEV protease to remove the N-terminal His tag and then used in phosphorylation assays in equal amounts in the presence of [γ -³³P]ATP and PknA. The MurC_{1T}, MurC_{2T}, MurC_{3T}, MurC_{4T}, MurC_{5T}, and MurC_{6T} mutant proteins corresponding to MurC_{362A}, MurC_{362A/365A}, MurC_{362A/365A/T51A}, MurC_{362A/365A/T51A/T120A}, MurC_{362A/365A/T51A/T120A/T167A}, and MurC_{362A/365A/T51A/T120A/T167A/T133A}, respectively, were separated by SDS-PAGE and stained with Coomassie Blue (*upper panel*), and the radioactive bands were revealed by autoradiography (*lower panel*).

was used. This technique has recently been proven to be a method of choice for characterizing post-translational modifications such as phosphorylation (18, 27, 37). LC-ESI/MS/MS was applied for the identification of phosphorylated peptides and for the localization of phosphorylation sites in MurC. Purified MurC was subjected to *in vitro* phosphorylation by PknA with non-radioactive ATP, prior to resolving on SDS-PAGE, and in-gel digestion with either trypsin or chymotrypsin. Phos-

phorylated amino acid residues were assigned by peptide fragmentation in MS/MS: *y* and *b* daughter ions containing one phosphothreonine were associated with a neutral loss of phosphoric acid ($-H_3PO_4$, *i.e.* -98 Da). As detailed in Table 3, analysis of tryptic and chymotryptic digests allowed the characterization of six phosphorylation sites in MurC corresponding to Thr⁵¹, Thr¹²⁰, Thr¹³³, Thr¹⁶⁷, Thr³⁶², and Thr³⁶⁵ (Figs. 1 and 4). These residues were found in the three structural domains of

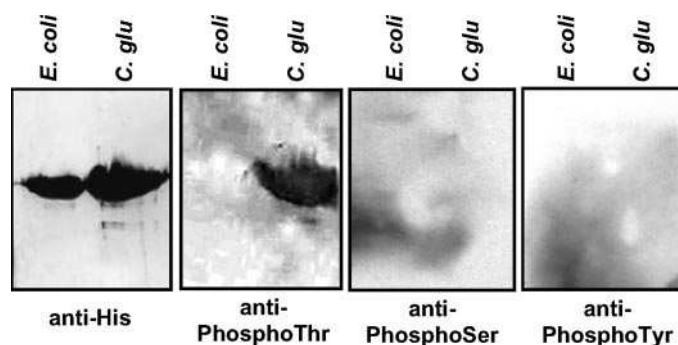


FIGURE 5. *In vivo* phosphorylation of MurC in *C. glutamicum*. Recombinant MurC purified from either *E. coli* or *C. glutamicum* strains were analyzed by SDS-PAGE and detected by immunoblotting using antibodies against His tag, phosphothreonine, phosphoserine, or phosphotyrosine residues.

the MurC protein, two of them, namely Thr¹²⁰ and Thr¹³³, being located very close to the ATP-binding site of the protein (¹²⁹GKT¹³¹). Thus, MS profiling coupled to *in vitro* kinase assays unambiguously demonstrated the phosphorylation of MurC by the PknA kinase. The fact that threonines but not serine residues were identified was consistent with our phosphoamino analysis (Fig. 2B).

Definitive identification and localization of the six threonine residues identified by mass spectrometry was achieved by site-directed mutagenesis by introducing mutations that prevent their specific phosphorylation. Thus, all the six threonine residues were sequentially replaced by alanine, yielding the MurC_{1T} to MurC_{6T} mutants. These mutant proteins were expressed, purified as His-tagged proteins in *E. coli* BL21(DE3) Star harboring the different mutant constructs (Table 1), and used in an *in vitro* kinase assay. After TEV protease cleavage of the His tag, the recombinant MurC phosphorylation site mutants were incubated with [γ -³³P]ATP and PknA. The proteins were separated by SDS-PAGE and analyzed by autoradiography. As shown in Fig. 4B (upper panel), equal amounts of MurC_{WT} or MurC mutants were used. Phosphorylation of the MurC_{6T} protein in which all six threonine residues were replaced by alanine appeared completely abolished, compared with phosphorylation of the intermediate mutants, as evidenced by the absence of a specific radioactive band (Fig. 4B, lower panel). Thus, these results unambiguously demonstrated that MurC without its six threonine phosphorylation sites has lost its ability to be phosphorylated by PknA, thus confirming the identification of all the sites of phosphorylation. An additional round of mass spectrometry analysis was also performed directly on the MurC_{6T} protein, which failed to identify any additional phosphate group that could eventually have arisen from a compensatory mechanism to the loss of the specific phosphorylation sites.

This type of analysis provided us the essential groundwork for mechanistic/functional studies of MurC regulation and demonstrated the efficiency of combining genetics and mass spectrometry analyses, with precise identification of phosphoacceptors, a prerequisite for a further understanding of the MurC mode of action. In particular, strains with defined mutations within the phosphorylation sites will be extremely helpful to establish the role of MurC phosphorylation-dependent regulation in corynebacterial growth and physiology.

In Vivo Phosphorylation of MurC in C. glutamicum—To assess the relevance of *in vitro* phosphorylation, the *in vivo* phosphorylation of MurC was also investigated in *C. glutamicum*. Therefore, to determine whether phosphorylation of MurC occurs on threonine residues *in vivo*, Western blot analysis was performed using specific anti-phosphothreonine, anti-phosphoserine, anti-phosphotyrosine, or anti-His antibodies. To overproduce and purify phosphorylated MurC, an His tag was attached at the C terminus of the MurC protein under the control of the *C. glutamicum* *divIVA* promoter (15) in plasmid pEDiv (Table 1), and the recombinant protein was expressed in *C. glutamicum*. Cultures of *E. coli*_{pTEVmurC} or *C. glutamicum*_{pEDivmurHis} overexpressing the His-tagged MurC protein were collected and lysed, and the soluble MurC was then purified to homogeneity as previously described (21). It was found that MurC purified from the *C. glutamicum* strain was only phosphorylated on threonine residues (Fig. 5). This was consistent with our phosphoamino acid content analyses and the *in vitro* identification of the MurC phosphorylation sites when phosphorylated by PknA. It clearly establishes that the murein ligase MurC is highly phosphorylated *in vivo* on threonines. In contrast, the anti-phosphothreonine antibodies did not react with recombinant MurC protein purified from *E. coli* thus confirming that phosphorylation of the *C. glutamicum* MurC did not occur following heterologous expression in *E. coli*, which thus confirmed its specific phosphorylation in *C. glutamicum* (Fig. 5).

MurC Phosphorylation Sites Are Critical for in Vitro and in Vivo Activity—The importance of the phosphorylation sites for MurC ligase activity was investigated *in vitro* by site-directed mutagenesis. The six threonine residues were replaced by alanine, and the catalytic activity of the wild-type and mutant purified MurC proteins was assayed by following the incorporation of L-[¹⁴C]alanine into the PG nucleotide precursor, as described under "Experimental Procedures." As shown in Fig. 6A, the activity of mutants MurC_{1T}, MurC_{2T}, and MurC_{3T}, corresponding to successive mutagenesis of Thr³⁶², Thr³⁶⁵, and Thr⁵¹ residues, was reduced by ~30–40% as compared with the wild-type level. Remarkably, the activity of the MurC_{4T} (T362A/T365A/T51A/T120A), MurC_{5T} (T362A/T365A/T51A/T120A/T167A), and MurC_{6T} (T362A/T365A/T51A/T120A/T167A/T133A) mutants appeared much more drastically altered and almost completely abolished in the six-threonine mutant (Fig. 6A). Moreover, to discern whether some threonines may be more important to murC activity than others individual mutagenesis was performed. The ligase activity of each individual mutant corresponding to MurC_{T51A}, MurC_{T120A}, MurC_{T133A}, MurC_{T167A}, MurC_{T362A}, and MurC_{T365A}, was of 72%, 29%, 44%, 79%, 26%, and 95% as compared with the wild-type level. Therefore, these results confirmed that Thr¹²⁰, Thr¹³³, and Thr³⁶² represent critical residues for MurC ligase activity, as already observed with the MurC_{1T} to MurC_{6T} mutants proteins (Fig. 6A).

These results confirmed that the overall activity of MurC relies on the presence of these threonine residues. In fact, one could imagine that replacing these critical residues present in all three characteristic domains of MurC could have a dramatic effect on the enzymatic activity of the ligase, and especially the

MurC Phosphorylation in *C. glutamicum*

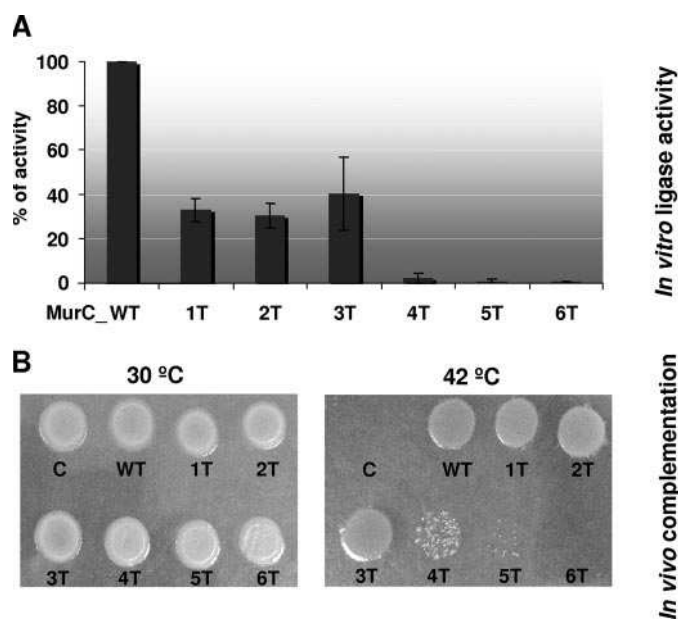


FIGURE 6. Effect of MurC phosphorylation site mutagenesis on *in vitro* and *in vivo* activity. *A*, *in vitro* assays of the wild-type and phosphorylation site mutant (1T to 6T) MurC proteins. Purified MurC proteins were assayed as described under "Experimental Procedures." Three independent experiments were performed, yielding similar results. The activity of the wild-type protein (here represented as 100%) was 2650 nmol/min/mg of protein, and those of the 1T, 2T, 3T, 4T, 5T, and 6T MurC mutants were 875, 800, 1050, 75, 20, and 10 nmol/min/mg of protein, respectively. *B*, *in vivo* functional complementation assays using an *E. coli* temperature-sensitive *murC* mutant strain. The pTrc99A plasmid vector and derivative plasmids expressing wild-type or mutated versions of the *C. glutamicum* MurC protein were transformed into the *E. coli* thermosensitive *murC* mutant strain H1119. Functional complementation was assayed by following the growth of the transformants at the permissive temperature of 30 °C (*left panel*), or at the non-permissive temperature of 42 °C (*right panel*).

mutagenesis of Thr¹³³, a key residue closed to the GKT motif necessary for the binding of ATP.

The activity of the MurC proteins was also tested *in vivo* using a functional assay based on complementation of the *E. coli* temperature-sensitive *murC* mutant strain H1119. This strain, which grows normally at 30 °C but lyses when shifted at the non-permissive temperature of 42 °C, was transformed with the pTrc99A plasmid, or the pTrc99MurC and pTrc99MurC1T, 2T, 3T, 4T, 5T, and 6T derivatives (Table 1) expressing wild-type or mutated forms of MurC. As shown in Fig. 6B, all types of transformants grew normally at the permissive temperature of 30 °C, but only those expressing the wild-type protein or the 1T, 2T, and 3T mutated proteins restored growth of the *E. coli* mutant at the temperature of 42 °C. No complementation by the plasmids expressing the 4T, 5T, and 6T mutants was observed (Fig. 6B).

A perfect correlation was thus observed between the *in vitro* and *in vivo* data, *i.e.* between the catalytic activity of these proteins and their ability to complement the *murC* temperature-sensitive *E. coli* mutant. Combined together, the results obtained with the different phosphorylation site mutants stress the importance of these threonine residues for MurC activity. These data not only suggest that these residues are important for a fully expressed activity of the MurC protein, due probably to their localization in critical regions of the enzyme, but also indicate, if we put forward the analysis, that the introduction of

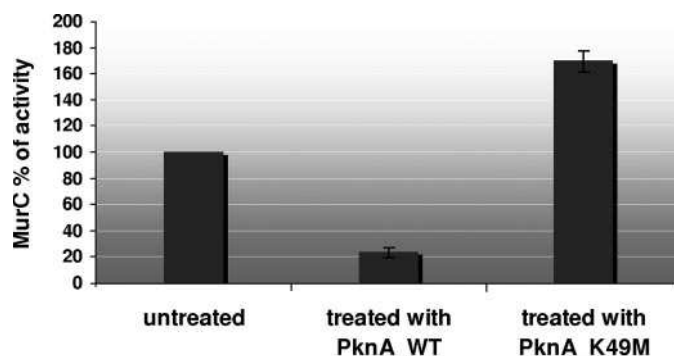


FIGURE 7. Comparative enzymatic activities of MurC phosphorylated versus non-phosphorylated. Purified wild-type MurC protein was phosphorylated either with PknA or PknA_K49M, and MurC ligase activity was assayed as described under "Experimental Procedures." Two independent protein preparations were assayed in triplicate, yielding similar results.

a negative charge due to the phosphorylation of threonines may have an impact on the regulation of the MurC enzyme activity.

Phosphorylation Negatively Modulates MurC Ligase Activity—Therefore, we assessed whether phosphorylation by PknA could have a direct effect on the regulation of the ligase activity of MurC. The *C. glutamicum* MurC protein purified from *E. coli* was pre-treated with either the PknA_WT kinase or the PknA_K49M kinase, yielding the phosphorylated and non-phosphorylated isoforms of MurC, respectively, which were subsequently tested for activity (Fig. 7). The PknA_K49M kinase, which is unable to autophosphorylate (Fig. 3) and therefore transphosphorylates its substrate, represented here an appropriate control, because one could imagine that the kinase itself could be responsible for the effect on MurC activity. Interestingly, we found that the ligase activity of the phosphorylated isoform of MurC was severely inhibited, representing ~30% of the wild-type activity, whereas no decrease of activity was observed following treatment by the PknA_K49M kinase (Fig. 7). An apparent increase of the MurC activity was observed in the latter case, suggesting that the presence of the mutant PknA protein, although inactive, stabilized in some way the MurC protein (protein-protein interactions) and reduced the slight loss of activity observed for MurC during the preincubation period. These results were consistent with the *in vitro* and *in vivo* analyses performed with the MurC mutants indicating that specific threonine residues were important in terms of enzymatic activity. Therefore, the PknA-mediated phosphorylation of MurC seems to represent a key mechanism for regulating/controlling its activity. Phosphorylation may rather modulate MurC activity on a fine-tuned level rather than a strict on/off mechanism. However, further work is needed to understand at a molecular level whether and how phosphorylation of MurC modifies the overall structure of this protein and negatively regulates the binding of L-alanine to UDP-MurNAc to generate the peptide moiety of the PG disaccharide peptide monomer unit.

We have previously shown that *C. glutamicum* PknA and PknB are key players in signal transduction pathways for the regulation of the cell shape and are both essential for sustaining corynebacterial growth (18). In this study, we extended our previous findings and demonstrated for the first time that PknA is able to specifically phosphorylate the essential murein ligase

enzyme MurC, both *in vitro* and *in vivo*, and that post-translational modifications appear to be a way to regulate its ligase activity. Moreover, due to the fact that corynebacteria are extensively used for industrial production of amino acids such as glutamate, these results may open the way to manipulation of the cell wall chemical structure to increase amino acid secretion. Furthermore, these findings could be useful to extend our present understanding of the *dcw* (division-cell wall biosynthesis) cluster in Gram-positive bacteria toward designing of new antimicrobial drugs targeting the cell wall metabolism processes like the one involving the Mur enzymes. Moreover, if these inhibitors are capable of interfering with these regulatory processes through selective inhibition of PknA or MurC phosphorylation, it may lead to the design of novel classes of inhibitors. Indeed, the development of STPK inhibitors and the expertise in designing such inhibitors for eukaryotic STPKs represent an intense research area and could be exploited for the development of new drugs, especially toward the pathogenic corynebacteria *Corynebacterium diphtheriae*.

REFERENCES

- Vicente, M., Hodgson, J., Massidda, O., Tonjum, T., Henriques-Normark, B., and Ron, E. Z. (2006) *FEMS Microbiol. Rev.* **30**, 841–852
- Barbosa, M. D., Ross, H. O., Hillman, M. C., Meade, R. P., Kurilla, M. G., and Pompliano, D. L. (2002) *Anal. Biochem.* **306**, 17–22
- Barreteau, H., Kovac, A., Boniface, A., Sova, M., Gobec, S., and Blanot, D. (2008) *FEMS Microbiol. Rev.* **32**, 168–207
- van Heijenoort, J. (1996) in *Escherichia Coli and Salmonella: Cellular and Molecular Biology* (Press, A., ed) pp. 1025–1034, ASM Press, Washington, DC
- van Heijenoort, J. (2001) *Nat. Prod. Rep.* **18**, 503–519
- Mengin-Lecreulx, D., Texier, L., Rousseau, M., and van Heijenoort, J. (1991) *J. Bacteriol.* **173**, 4625–4636
- Kotnik, M., Anderlueh, P. S., and Prezeli, A. (2007) *Curr. Pharm. Des.* **13**, 2283–2309
- El Zoeiby, A., Sanschagrín, F., and Levesque, R. C. (2003) *Mol. Microbiol.* **47**, 1–12
- Ehmann, D. E., Demeritt, J. E., Hull, K. G., and Fisher, S. L. (2004) *Biochim. Biophys. Acta* **1698**, 167–174
- Zawadzke, L. E., Norcia, M., Desbonnet, C. R., Wang, H., Freeman-Cook, K., and Dougherty, T. J. (2008) *Assay Drug Dev. Technol.* **6**, 95–103
- Strancar, K., Blanot, D., and Gobec, S. (2006) *Bioorg. Med. Chem. Lett.* **16**, 343–348
- Strancar, K., Boniface, A., Blanot, D., and Gobec, S. (2007) *Arch. Pharm. (Weinheim)* **340**, 127–134
- Hermann, T. (2003) *J. Biotechnol.* **104**, 155–172
- Daniel, R. A., and Errington, J. (2003) *Cell* **113**, 767–776
- Letek, M., Ordonez, E., Vaquera, J., Margolin, W., Flardh, K., Mateos, L. M., and Gil, J. A. (2008a) *J. Bacteriol.* **190**, 3283–3292
- Letek, M., Fiuza, M., Ordonez, E., Villadangos, A. F., Ramos, A., Mateos, L. M., and Gil, J. A. (2008b) *Antonie Van Leeuwenhoek* **94**, 99–109
- Bendt, A. K., Burkovski, A., Schaffer, S., Bott, M., Farwick, M., and Hermann, T. (2003) *Proteomics* **3**, 1637–1646
- Fiuza, M., Canova, M. J., Zanella-Cleon, I., Becchi, M., Cozzzone, A. J., Mateos, L. M., Kremer, L., Gil, J. A., and Molle, V. (2008) *J. Biol. Chem.* **283**, 18099–18112
- Thakur, M., and Chakraborti, P. K. (2008) *Biochem. J.* **415**, 27–33
- Mateos, L. M., Schafer, A., Kalinowski, J., Martin, J. F., and Puhler, A. (1996) *J. Bacteriol.* **178**, 5768–5775
- Molle, V., Brown, A. K., Besra, G. S., Cozzzone, A. J., and Kremer, L. (2006) *J. Biol. Chem.* **281**, 30094–30103
- Amann, E., Ochs, B., and Abel, K. J. (1988) *Gene (Amst.)* **69**, 301–315
- Lugtenberg, E. J., and v Schijndel-van Dam, A. (1972) *J. Bacteriol.* **110**, 35–40
- Liger, D., Masson, A., Blanot, D., van Heijenoort, J., and Parquet, C. (1995) *Eur. J. Biochem.* **230**, 80–87
- Bouhss, A., Mengin-Lecreulx, D., Blanot, D., van Heijenoort, J., and Parquet, C. (1997) *Biochemistry* **36**, 11556–11563
- Deva, T., Baker, E. N., Squire, C. J., and Smith, C. A. (2006) *Acta Crystallogr. D. Biol. Crystallogr.* **62**, 1466–1474
- Canova, M. J., Veyron-Churlet, R., Zanella-Cleon, I., Cohen-Gonsaud, M., Cozzzone, A. J., Becchi, M., Kremer, L., and Molle, V. (2008) *Proteomics* **8**, 521–533
- Molle, V., Kremer, L., Girard-Blanc, C., Besra, G. S., Cozzzone, A. J., and Prost, J. F. (2003) *Biochemistry* **42**, 15300–15309
- Peirs, P., De Wit, L., Braibant, M., Huygen, K., and Content, J. (1997) *Eur. J. Biochem.* **244**, 604–612
- Huse, M., and Kuriyan, J. (2002) *Cell* **109**, 275–282
- Johnson, L. N., Noble, M. E., and Owen, D. J. (1996) *Cell* **85**, 149–158
- Dasgupta, A., Datta, P., Kundu, M., and Basu, J. (2006) *Microbiology* **152**, 493–504
- Boitel, B., Ortiz-Lombardia, M., Duran, R., Pompeo, F., Cole, S. T., Cervenansky, C., and Alzari, P. M. (2003) *Mol. Microbiol.* **49**, 1493–1508
- Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) *Science* **241**, 42–52
- Motley, S. T., and Lory, S. (1999) *Infect. Immun.* **67**, 5386–5394
- Villarino, A., Duran, R., Wehenkel, A., Fernandez, P., England, P., Brodin, P., Cole, S. T., Zimny-Arndt, U., Jungblut, P. R., Cervenansky, C., and Alzari, P. M. (2005) *J. Mol. Biol.* **350**, 953–963
- Molle, V., Zanella-Cleon, I., Robin, J. P., Mallejac, S., Cozzzone, A. J., and Becchi, M. (2006) *Proteomics* **6**, 3754–3766
- Schafer, A., Kalinowski, J., Simon, R., Seep-Feldhaus, A. H., and Puhler, A. (1990) *J. Bacteriol.* **172**, 1663–1666
- Santamaria, R. I., Martin, J. F., and Gil, J. A. (1987) *Gene (Amst.)* **56**, 199–208
- Cohen-Gonsaud, M., Barthe, P., Pommier, F., Harris, R., Driscoll, P. C., Keep, N. H., and Roumestand, C. (2004) *J. Biomol. NMR* **30**, 373–374

Chapitre **3**

Chapitre 3 : Discussion et Conclusion

Rv2175 est une protéine d'attachement à l'ADN régulée par phosphorylation via PknL

La phosphorylation via les protéine-kinases a pour but de moduler diverses fonctions, et intervient, par exemple, dans la régulation de différents facteurs de transcription, la localisation cellulaire, l'activité enzymatique, etc. Les protéine-kinases de *M. tuberculosis* sont impliquées dans ces différentes voies de transduction du signal et nos travaux se sont inscrits dans la caractérisation de ces kinases et de leur rôle physiologique.

Tout d'abord, les différentes études bioinformatiques, par spectrométrie de masse et de caractérisation biochimique menées sur PknL, nous ont permis de confirmer que cette kinase faisait partie de la famille des Ser/Thr kinases à domaines de Hanks, caractéristiques des STPKs. En effet, nous avons mis en évidence le rôle du résidu lysyle catalytique en position 48 essentiel pour son activité d'autophosphorylation, et détecté par spectrométrie de masse les cinq sites de phosphorylation de PknL dont deux situés au niveau de la boucle d'activation, régulant ainsi son activité. Parmi les onze STPKs présentes chez *M. tuberculosis*, neuf possèdent un domaine transmembranaire suivi d'un domaine extra-cellulaire. Cette région extra-cellulaire peut présenter plusieurs motifs protéiques connus (domaine PASTA chez PknB, motif thioredoxin-like chez PknE), ou adopter une structure particulière (hélice β chez PknD). Bien que son rôle soit encore mal expliqué, cette portion extra-cellulaire semble impliquée dans la détection de signaux externes et la transduction de ces signaux. PknL possède un domaine transmembranaire qui la prédispose à s'ancrer au niveau de la membrane mais ne présente pas de domaine extracellulaire. Par conséquent, PknL ne serait apparemment pas impliquée dans la transduction des signaux extracellulaires mais répondrait à d'autres types de signaux et présenterait donc un mode d'activation différent.

D'une manière générale, la régulation de l'activité des protéine-kinases passe par le contrôle de l'accès au site catalytique, et/ou au site de liaison du substrat, ou encore par le réarrangement d'éléments structuraux impliqués dans la catalyse ou la reconnaissance du substrat. Chez les procaryotes, comme chez les eucaryotes, il existe un grand nombre de mécanismes de régulation impliquant des réactions de phosphorylation/ déphosphorylation. Chez *M. tuberculosis*, des études par spectrométrie de masse ont permis d'identifier les sites de phosphorylation chez PknA, B, D, E, F et H (Duran *et al.*, 2005 ; Molle *et al.*, 2006). Ces études ont permis de montrer que le nombre de sites variait selon la kinase, et divers sites de

phosphorylation ont pu être identifiés au niveau de la boucle d'activation de PknB, D, E et F. En effet, la phosphorylation de la boucle d'activation est un élément de contrôle majeur pour le passage de la conformation inactive à la conformation active de nombreuses kinases. L'étude de la régulation de l'activité de PknB par l'équipe de Pedro Alzari (Boitel *et al.*, 2003) montre que l'état de phosphorylation des thréonines T171 et T173 de la boucle d'activation est déterminant pour son activité kinase. Cette étude confirme que ces deux résidus thréonyles sont indispensables à l'activité d'autophosphorylation de la kinase et sont impliqués de manière équivalente dans l'activation de la kinase. Dans notre étude, nous avons identifié cinq sites de phosphorylation chez PknL, dont deux situés dans la boucle d'activation (T173 et T175), correspondant à T171 et T173 chez PknB. Cependant, si nous mettons en évidence que les deux sites sont nécessaires pour l'activité totale d'autophosphorylation, nous soulignons aussi le fait que la phosphorylation du résidu T173 est plus importante que celle de T175 dans le processus d'activation de la kinase, mettant ainsi à jour un mécanisme d'activation et/ou de régulation différent de celui caractérisé pour PknB. Cette dissemblance d'avec PknB pourrait s'expliquer par des différences de structure au niveau du site actif et/ou au niveau de la protéine entière. Les études sur la structure de PknB ont également permis de mettre en évidence que, contrairement aux kinases eucaryotes, la boucle d'activation de PknB était désordonnée. Les résidus thréonyles phosphorylés interagissent avec un cluster d'arginines composé des résidus R55, R137 et R161, ce qui maintient la boucle en conformation « active ». Ces arginines sont conservées chez PknL et il est donc possible que ce cluster participe également au maintien la boucle d'activation de PknL en conformation « active » lorsque celle-ci est phosphorylée.

Comme pour PknB, D, E et F, nous avons identifié un site de phosphorylation dans le domaine juxtamembranaire de PknL. Bien que cette région ne semble pas nécessaire à l'activité d'autophosphorylation de PknB ou de PknF, le fait d'identifier dans plusieurs STPKs des sites dans ce domaine laisse à spéculer que ce domaine juxtamembranaire pourrait jouer un rôle dans l'activation de la kinase ou dans sa stabilité comme cela a été démontré chez d'autres STPKs. La phosphorylation de ce domaine chez PknL pourrait avoir un rôle, non pas dans la régulation de l'activité d'autophosphorylation de la kinase, mais dans la régulation du recrutement d'autres partenaires protéiques au sein d'une cascade de signalisation.

La structure de PknL n'est pas encore déterminée. Cependant les études structurales menées par les équipes de Pedro Alzari et de Tom Alder sur PknB, D et E, permettent de

discuter du mode d'activation de ces kinases. Les différentes études ont montré que ces trois kinases se trouvent sous forme d'homodimères en « dos à dos », et que l'homodimérisation serait impliquée dans les mécanismes de régulation de la kinase. La résolution de la structure de PknB sous forme dimérique a également permis de mettre en évidence huit résidus conservés à l'interface entre les deux monomères, dont quatre directement impliqués dans l'interaction monomère-monomère. Ces huit résidus sont quasiment tous conservés chez PknL. Cette observation, bien que basée uniquement sur la séquence primaire, permet d'émettre l'hypothèse d'une régulation de PknL par dimérisation.

La spectrométrie de masse est une technologie performante que nous avons développée et adaptée au laboratoire afin d'identifier les sites de phosphorylation des différentes kinases et substrats. Nous avons identifié cinq sites pour PknL. Cependant, il n'est pas exclu que d'autres sites soient présents mais que nous ne soyons pas en mesure de les détecter. Tout d'abord, le recouvrement de la séquence de PknL ne se fait qu'à hauteur de 75%. Il se peut également qu'il existe des sites de phosphorylation principaux, phosphorylés systématiquement, et des sites secondaires. C'est, par exemple, le cas pour les sites de la boucle d'activation de PknB pour laquelle T171 et T173 correspondent aux sites principaux, alors que S169 et T179 sont des sites secondaires qui ne se trouvent pas systématiquement phosphorylés (Boitel *et al.*, 2003). Il se peut, enfin, que la proportion de peptide phosphorylé lors de l'analyse de spectrométrie de masse soit trop faible pour permettre l'identification des sites.

En ce qui concerne le rôle *in vivo* de PknL, aucune information n'a été rapportée à ce jour. Cependant, PknL est présente chez les mycobactéries, les corynébactéries et les streptomycètes. On la retrouve également chez les organismes possédant un nombre réduit de kinases (*M. leprae* et *C. glutamicum*). Cette ubiquité semble indiquer un rôle important de PknL dans la survie et le développement des bactéries. En revanche, des expériences d'inactivation du gène *pknL* dans la cellule réalisées chez *C. glutamicum* et *M. tuberculosis* ont montré le caractère non essentiel de PknL pour le développement des microorganismes.

Dans le but de mettre en évidence le ou les substrats de PknL, nous nous sommes concentrés, dans un premier temps, sur son environnement génétique. En effet, il a été précédemment montré que les gènes codant des substrats de kinases pouvaient être localisés dans un environnement proche des gènes codant les kinases elles-mêmes. Par exemple, le gène codant Rv1747 est situé à côté du gène de PknF ; de même pour le gène codant PknH

qui est, lui, situé dans le même opéron que EmbR, ainsi que pour le couple *pknB/pbpA*. Notre étude s'est donc logiquement portée sur la protéine Rv2175c dont le gène est situé en amont de celui de PknL. Nous avons ainsi mis en évidence que Rv2175c était un substrat spécifique de PknL *in vitro*. Le site de phosphorylation a été caractérisé par spectrométrie de masse comme étant le résidu Thr9. Nous avons montré, d'une part, que l'autophosphorylation de PknL était un prérequis à la phosphorylation de Rv2175c et que, d'autre part, la phosphorylation de Rv2175c était dépendante de l'état de phosphorylation de la boucle d'activation de la kinase en général, et du résidu T173 en particulier. Les études préliminaires d'analyse bioinformatique de la séquence primaire de Rv2175c ont montré que cette protéine possédait un domaine similaire au domaine hélice-tour-hélice (HTH), semblant classer cette protéine de rôle inconnu dans la famille des protéines capables de fixer l'ADN. Afin de déterminer la fonction de cette protéine, la structure de Rv2175c a été résolue par RMN. Cette protéine possède un domaine N-terminal en hélice-tour-hélice correspondant au probable motif de liaison à l'ADN, et un domaine C-terminal, probablement régulateur. Le site unique de phosphorylation (T9) se situe dans une portion flexible à l'extrémité du domaine N-terminal. Des expériences d'anisotropie de fluorescence et de gel retard nous ont ensuite permis de confirmer l'activité de fixation à l'ADN de Rv2175c et de supposer un rôle probable de régulateur transcriptionnel.

D'autres facteurs de transcription phosphorylés par les STPKs ont été caractérisés chez *M. tuberculosis*. En effet, la phosphorylation d'EmbR par PknH est impliquée dans la régulation de la synthèse de la paroi bactérienne ou, par exemple, la phosphorylation de VirS par PknK entraîne l'activation de l'opéron *mymA*. La phosphorylation augmente l'affinité du régulateur transcriptionnel pour l'ADN dans les deux cas. A l'inverse, nous montrons que la phosphorylation de Rv2175c sur le résidu T9 inhibe son pouvoir de fixation à l'ADN. Bien que les mécanismes de cette inhibition ne soient pas encore totalement élucidés, il se peut que l'addition d'un groupement phosphate entraîne un phénomène de répulsion de charge entre la protéine et l'ADN cible. Il a déjà été montré que les protéines de fixation à l'ADN en général et, plus particulièrement, les facteurs de transcription bactériens, se fixent à l'ADN sous forme d'homodimères. Il est donc possible que la phosphorylation de Rv2175c empêche la dimérisation de la protéine et, par là, la fixation à l'ADN.

De plus, il est important de mentionner que la portion N-terminale flexible portant le site de phosphorylation T9 est uniquement présente chez les homologues de Rv2175c dans les souches de *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microtii* et *M. canetti*, alors que cette région est absente chez *M. leprae* et *M. smegmatis*. Cette particularité pourrait signifier que la

régulation de Rv2175c ferait appel à un autre mécanisme que la phosphorylation chez *M. leprae* et *M. smegmatis*, car les derniers résultats ont montré que Rv2175c de *M. smegmatis* n'était pas phosphorylé, tout du moins avec la kinase homologue à PknF chez *M. smegmatis*. Le rôle physiologique du couple PknL/Rv2175c n'est pas encore élucidé, même si le fait que le cluster de division *dcw* adjacent sur le génome représente une cible à considérer. Des expériences de « chIP-to-chip » devraient, dans un premier temps, nous permettre de mettre en évidence les différents gènes régulés par Rv2175c. Une fois les cibles génétiques identifiées, la génération de souches isogéniques avec le gène Rv2175c muté soit dans une forme empêchant la phosphorylation (T9A), soit dans une forme mimant la phosphorylation (T9D), pourrait ensuite permettre d'appréhender le rôle de la phosphorylation sur la régulation de Rv2175c.

La phosphorylation de la chaperonne GroEL1 est-elle un mode de régulation spécifique aux mycobactéries pathogènes?

La mise en évidence, au cours de cette étude, de la phosphorylation de la chaperonne GroEL1 de *M. tuberculosis* nous a permis de caractériser un nouveau couple kinase/substrat. Alors que la phosphorylation de GroEL a déjà été montrée chez *Escherichia coli* (Sherman & Goldberg, 1994), *Thiobacillus ferrooxidans* (Seeger *et al.*, 1996) et *Streptomyces granaticolors* (Bobek *et al.*, 2004), c'est la première fois que ce type de modification post-traductionnelle est décrite chez une chaperonne de *M. tuberculosis*.

Tout d'abord, des tests de phosphorylation *in vitro* ont montré que GroEL1 pouvait être phosphorylée par différentes STPKs, mais semble préférentiellement interagir avec PknF. En effet, de précédentes études ont montré que PknF possédait une activité d'autophosphorylation, et six sites de phosphorylation ont été identifiés (Duran *et al.*, 2005). A l'instar de PknB, deux sites de phosphorylation ont été identifiés dans le segment juxtamembranaire, mais ne semblent pas intervenir dans la régulation de l'activité de la kinase. En revanche, la phosphorylation des deux sites T173 et T175 présents dans la boucle d'activation est indispensable à l'activité optimale d'autophosphorylation de la kinase. De plus, de même que pour PknL, la thréonine173 joue un rôle prépondérant dans le mécanisme d'activation de la kinase. Cette similarité avec ce que nous avons montré pour PknL suggère un mécanisme d'autophosphorylation commun aux deux kinases, et qui différerait de celui de PknB.

Préalablement caractérisée comme étant capable de phosphoryler les domaines FHA de Rv1747 (Molle *et al.*, 2004) et de Rv0020 (Grunder *et al.*, 2005), PknF apparaît ainsi également capable de phosphoryler des protéines dépourvues de domaines FHA, et ouvre par conséquent un nouvel aspect de la régulation kinase/substrat. De même que pour PknL, nous mettons en évidence que pour le recrutement et la phosphorylation de GroEL1, PknF doit être activée, et que les résidus T173 et T175 de la boucle d'activation doivent être phosphorylés. Des analyses par spectrométrie de masse nous ont permis d'identifier deux sites de phosphorylation correspondant à T25 et T54. L'analyse des profils de phosphorylation des différents mutants ponctuels T25A et T54A a montré que ces deux sites ne sont pas équivalents vis-à-vis de la phosphorylation via PknF. Alors que la mutation du site T54 ne provoque pas de diminution notable de la phosphorylation de GroEL1, l'absence du résidu thréonyle 25 entraîne une perte de phosphorylation quasi-totale. Par conséquent, il apparaît possible que T25 représente un site de phosphorylation essentiel à la phosphorylation optimale de GroEL1.

Le rôle de la phosphorylation dans la régulation de l'activité de GroEL1 n'est, à ce jour, pas encore totalement élucidé. En revanche, plusieurs hypothèses peuvent être formulées. Tout d'abord, chez *E. coli* la phosphorylation de GroEL1 induite par un choc thermique à 42°C altère la fixation de la chaperonne sur les protéines non repliées et facilite ainsi leur dissociation (Sherman & Goldberg, 1994). Bien que GroEL1 de *M. tuberculosis* ne présente qu'une faible activité chaperonne, elle possède tout de même la capacité de protéger les protéines de la dégradation (Qamra *et al.*, 2004). Il est donc possible que la phosphorylation de GroEL1 de *M. tuberculosis* influe sur son activité chaperonne. Différents tests sont actuellement en cours pour tenter de mettre en évidence le rôle de la phosphorylation de GroEL1 sur son activité protectrice et/ou sur son rôle dans le repliement protéique.

D'autre part, les chaperonnes de *M. tuberculosis* sont connues pour être impliquées dans différentes voies de signalisation intercellulaire induisant des réactions pro-inflammatoires. De plus, une récente étude de l'équipe de Brian Henderson a montré que GroEL1 était responsable de l'activation de la synthèse de cytokines par le monocyte humain. Plus précisément, cette équipe a mis en évidence que la stimulation des monocytes se faisait par interaction entre les récepteurs CD14 du monocyte et le domaine équatorial de GroEL1, se traduisant ainsi par la production de cytokines pro-inflammatoires (Tormay *et al.*, 2005). Il est intéressant de remarquer que les deux sites de phosphorylation identifiés sur GroEL1 se trouvent au niveau du domaine équatorial de la protéine. Par conséquent, de futures analyses

in vitro et *in vivo* devraient permettre d'établir s'il existe un lien entre la phosphorylation de GroEL1 et l'activation des monocytes.

Enfin, alors que chez *M. tuberculosis*, GroEL1 (GroEL1_{Mtb}) semble être impliquée dans la stimulation de la réaction pro-inflammatoire, chez *M. smegmatis* cette chaperonne (GroEL1_{Msm}) interagit avec l'enzyme de condensation KasA impliquée dans la synthèse des acides mycoliques et interviendrait dans la maturation des biofilms. GroEL1 semble donc jouer des rôles différents selon les espèces et il est fort probable que la phosphorylation soit impliquée dans ces différences. En effet, alors que GroEL1_{Mtb} est phosphorylée, nous avons montré que GroEL1_{Msm} n'était pas phosphorylée par l'homologue de PknF chez *M. smegmatis*. Une des raisons pour lesquelles GroEL1_{Msm} pouvait ne pas être phosphorylable tient au fait qu'un des résidus (T25) identifiés chez GroEL1_{Mtb} comme site de phosphorylation n'était pas présent chez GroEL1_{Msm} et était remplacé par un résidu alanine. Par conséquent, nous avons entrepris de muter ce résidu en un résidu thréonyle et, de ce fait, la phosphorylation a pu être restaurée chez GroEL1_{Msm}. Ces résultats semblent confirmer que la phosphorylation serait un mode de régulation propre aux chaperonnes appartenant à la famille des mycobactéries pathogènes et possédant, de ce fait, ce site apparemment primordial pour une régulation via phosphorylation.

En conclusion, l'ensemble de ces travaux nous a permis d'approfondir l'étude génétique et biochimique des couples kinase/substrat chez les mycobactéries et de mieux appréhender à l'échelle moléculaire le rôle de la phosphorylation dans la régulation de la physiologie de ce genre bactérien qui revêt une importance particulière en termes de santé publique.

BIBLIOGRAPHIE

- Alderwick, L. J., V. Molle, L. Kremer, A. J. Cozzone, T. R. Dafforn, G. S. Besra and K. Futterer, (2006) Molecular structure of EmbR, a response element of Ser/Thr kinase signaling in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* **103**: 2558-2563.
- Allen, G. S., K. Steinhauer, W. Hillen, J. Stulke and R. G. Brennan, (2003) Crystal structure of HPr kinase/phosphatase from *Mycoplasma pneumoniae*. *J Mol Biol* **326**: 1203-1217.
- Av-Gay, Y. and M. Everett, (2000) The eukaryotic-like Ser/Thr protein kinases of *Mycobacterium tuberculosis*. *Trends Microbiol* **8**: 238-244.
- Av-Gay, Y., S. Jamil and S. J. Drews, (1999) Expression and characterization of the *Mycobacterium tuberculosis* serine/threonine protein kinase PknB. *Infect Immun* **67**: 5676-5682.
- Bach, H., D. Wong and Y. Av-Gay, (2009) *Mycobacterium tuberculosis* PtkA is a novel protein tyrosine kinase whose substrate is PtpA. *Biochem J* **420**: 155-160.
- Bender, M. H., R. T. Cartee and J. Yother, (2003) Positive correlation between tyrosine phosphorylation of CpsD and capsular polysaccharide production in *Streptococcus pneumoniae*. *J Bacteriol* **185**: 6057-6066.
- Bendt, A. K., A. Burkovski, S. Schaffer, M. Bott, M. Farwick and T. Hermann, (2003) Towards a phosphoproteome map of *Corynebacterium glutamicum*. *Proteomics* **3**: 1637-1646.
- Bidle, K. A., (2003) Differential expression of genes influenced by changing salinity using RNA arbitrarily primed PCR in the archaeal halophile *Haloferax volcanii*. *Extremophiles* **7**: 1-7.

- Bobek, J., P. Halada, J. Angelis, J. Vohradsky and K. Mikulik, (2004) Activation and expression of proteins during synchronous germination of aerial spores of *Streptomyces granaticolor*. *Proteomics* **4**: 3864-3880.
- Boel, G., I. Mijakovic, A. Maze, S. Poncet, M. K. Taha, M. Larribe, E. Darbon, A. Khemiri, A. Galinier and J. Deutscher, (2003) Transcription regulators potentially controlled by HPr kinase/phosphorylase in Gram-negative bacteria. *J Mol Microbiol Biotechnol* **5**: 206-215.
- Boitel, B., M. Ortiz-Lombardia, R. Duran, F. Pompeo, S. T. Cole, C. Cervenansky and P. M. Alzari, (2003) PknB kinase activity is regulated by phosphorylation in two Thr residues and dephosphorylation by PstP, the cognate phospho-Ser/Thr phosphatase, in *Mycobacterium tuberculosis*. *Mol Microbiol* **49**: 1493-1508.
- Bossemeyer, D., (1995) Protein kinases--structure and function. *FEBS Lett* **369**: 57-61.
- Boylan, S. A., A. Rutherford, S. M. Thomas and C. W. Price, (1992) Activation of *Bacillus subtilis* transcription factor sigma B by a regulatory pathway responsive to stationary-phase signals. *J Bacteriol* **174**: 3695-3706.
- Brennan, P. J. and H. Nikaido, (1995) The envelope of mycobacteria. *Annu Rev Biochem* **64**: 29-63.
- Brychzy, A., T. Rein, K. F. Winklhofer, F. U. Hartl, J. C. Young and W. M. Obermann, (2003) Cofactor Tpr2 combines two TPR domains and a J domain to regulate the Hsp70/Hsp90 chaperone system. *EMBO J* **22**: 3613-3623.
- Carrera, A. C., K. Alexandrov and T. M. Roberts, (1993) The conserved lysine of the catalytic domain of protein kinases is actively involved in the phosphotransfer reaction and not required for anchoring ATP. *Proc Natl Acad Sci U S A* **90**: 442-446.
- Chaba, R., M. Raje and P. K. Chakraborti, (2002) Evidence that a eukaryotic-type serine/threonine protein kinase from *Mycobacterium tuberculosis* regulates morphological changes associated with cell division. *Eur J Biochem* **269**: 1078-1085.
- Chang, C. and E. M. Meyerowitz, (1995) The ethylene hormone response in *Arabidopsis*: a eukaryotic two-component signaling system. *Proc Natl Acad Sci U S A* **92**: 4129-4133.

- Check, E., (2007) Unlikely partners tackle TB funding woes. *Nat Med* **13**: 265.
- Chopra, P., B. Singh, R. Singh, R. Vohra, A. Koul, L. S. Meena, H. Koduri, M. Ghildiyal, P. Deol, T. K. Das, A. K. Tyagi and Y. Singh, (2003) Phosphoprotein phosphatase of *Mycobacterium tuberculosis* dephosphorylates serine-threonine kinases PknA and PknB. *Biochem Biophys Res Commun* **311**: 112-120.
- Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaiia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead and B. G. Barrell, (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**: 537-544.
- Collins, R. F., K. Beis, B. R. Clarke, R. C. Ford, M. Hulley, J. H. Naismith and C. Whitfield, (2006) Periplasmic protein-protein contacts in the inner membrane protein Wzc form a tetrameric complex required for the assembly of *Escherichia coli* group 1 capsules. *J Biol Chem* **281**: 2144-2150.
- Core, L. and M. Perego, (2003) TPR-mediated interaction of RapC with ComA inhibits response regulator-DNA binding for competence development in *Bacillus subtilis*. *Mol Microbiol* **49**: 1509-1522.
- Cortay, J. C., C. Rieul, B. Duclos and A. J. Cozzone, (1986) Characterization of the phosphoproteins of *Escherichia coli* cells by electrophoretic analysis. *Eur J Biochem* **159**: 227-237.
- Cowley, S., M. Ko, N. Pick, R. Chow, K. J. Downing, B. G. Gordhan, J. C. Betts, V. Mizrahi, D. A. Smith, R. W. Stokes and Y. Av-Gay, (2004) The *Mycobacterium tuberculosis* protein serine/threonine kinase PknG is linked to cellular glutamate/glutamine levels and is important for growth *in vivo*. *Mol Microbiol* **52**: 1691-1702.

- Cox, S., E. Radzio-Andzelm and S. S. Taylor, (1994) Domain movements in protein kinases. *Curr Opin Struct Biol* **4**: 893-901.
- Cozzone, A. J., (1998a) Post-translational modification of proteins by reversible phosphorylation in prokaryotes. *Biochimie* **80**: 43-48.
- Cozzone A. J., (1998b) Regulation of acetate metabolism by protein phosphorylation in enteric bacteria. *Annu Rev Microbiol.* **52**:127-64.
- Cozzone, A. J., (2005) Role of protein phosphorylation on serine/threonine and tyrosine in the virulence of bacterial pathogens. *J Mol Microbiol Biotechnol* **9**: 198-213.
- Curry, J. M., R. Whalan, D. M. Hunt, K. Gohil, M. Strom, L. Rickman, M. J. Colston, S. J. Smerdon and R. S. Buxton, (2005) An ABC transporter containing a forkhead-associated domain interacts with a serine-threonine protein kinase and is required for growth of *Mycobacterium tuberculosis* in mice. *Infect Immun* **73**: 4471-4477.
- Dasgupta, A., P. Datta, M. Kundu and J. Basu, (2006) The serine/threonine kinase PknB of *Mycobacterium tuberculosis* phosphorylates PBPA, a penicillin-binding protein required for cell division. *Microbiology* **152**: 493-504.
- Deol, P., R. Vohra, A. K. Saini, A. Singh, H. Chandra, P. Chopra, T. K. Das, A. K. Tyagi and Y. Singh, (2005) Role of *Mycobacterium tuberculosis* Ser/Thr kinase PknF: implications in glucose transport and cell division. *J Bacteriol* **187**: 3415-3420.
- Deutscher, J., C. Fischer, V. Charrier, A. Galinier, C. Lindner, E. Darbon and V. Dossonnet, (1997) Regulation of carbon metabolism in gram-positive bacteria by protein phosphorylation. *Folia Microbiol (Praha)* **42**: 171-178.
- Deutscher, J., U. Kessler and W. Hengstenberg, (1985) Streptococcal phosphoenolpyruvate: sugar phosphotransferase system: purification and characterization of a phosphoprotein phosphatase which hydrolyzes the phosphoryl bond in seryl-phosphorylated histidine-containing protein. *J Bacteriol* **163**: 1203-1209.

- Deutscher, J. and M. H. Saier, Jr., (1983) ATP-dependent protein kinase-catalyzed phosphorylation of a seryl residue in HPr, a phosphate carrier protein of the phosphotransferase system in *Streptococcus pyogenes*. *Proc Natl Acad Sci U S A* **80**: 6790-6794.
- Doublet, P., C. Grangeasse, B. Obadia, E. Vaganay and A. J. Cozzone, (2002) Structural organization of the protein-tyrosine autokinase Wzc within *Escherichia coli* cells. *J Biol Chem* **277**: 37339-37348.
- Duclos, B., C. Grangeasse, E. Vaganay, M. Riberty and A. J. Cozzone, (1996) Autophosphorylation of a bacterial protein at tyrosine. *J Mol Biol* **259**: 891-895.
- Duclos, B., S. Marcandier and A. J. Cozzone, (1991) Chemical properties and separation of phosphoamino acids by thin-layer chromatography and/or electrophoresis. *Methods Enzymol* **201**: 10-21.
- Duran, R., A. Villarino, M. Bellinzoni, A. Wehenkel, P. Fernandez, B. Boitel, S. T. Cole, P. M. Alzari and C. Cervenansky, (2005) Conserved autophosphorylation pattern in activation loops and juxtamembrane regions of *Mycobacterium tuberculosis* Ser/Thr protein kinases. *Biochem Biophys Res Commun* **333**: 858-867.
- Durocher, D. and S. P. Jackson, (2002) The FHA domain. *FEBS Lett* **513**: 58-66.
- Dye, C., (2006) Global epidemiology of tuberculosis. *Lancet* **367**: 938-940.
- Fernandez, P., B. Saint-Joanis, N. Barilone, M. Jackson, B. Gicquel, S. T. Cole and P. M. Alzari, (2006) The Ser/Thr protein kinase PknB is essential for sustaining mycobacterial growth. *J Bacteriol* **188**: 7778-7784.
- Fieulaine, S., S. Morera, S. Poncet, I. Mijakovic, A. Galinier, J. Janin, J. Deutscher and S. Nessler, (2002) X-ray structure of a bifunctional protein kinase in complex with its protein substrate HPr. *Proc Natl Acad Sci U S A* **99**: 13437-13441.
- Fieulaine, S., S. Morera, S. Poncet, V. Monedero, V. Gueguen-Chaignon, A. Galinier, J. Janin, J. Deutscher and S. Nessler, (2001) X-ray structure of HPr kinase: a bacterial protein kinase with a P-loop nucleotide-binding domain. *EMBO J* **20**: 3917-3927.

- Frieden, T. R., T. R. Sterling, S. S. Munsiff, C. J. Watt and C. Dye, (2003) Tuberculosis. *Lancet* **362**: 887-899.
- Garnak, M. and H. C. Reeves, (1979) Phosphorylation of Isocitrate dehydrogenase of *Escherichia coli*. *Science* **203**:1111-2.
- Gay, L. M., H. L. Ng and T. Alber, (2006) A conserved dimer and global conformational changes in the structure of apo-PknE Ser/Thr protein kinase from *Mycobacterium tuberculosis*. *J Mol Biol* **360**: 409-420.
- Good, M. C., A. E. Greenstein, T. A. Young, H. L. Ng and T. Alber, (2004) Sensor domain of the *Mycobacterium tuberculosis* receptor Ser/Thr protein kinase, PknD, forms a highly symmetric beta propeller. *J Mol Biol* **339**: 459-469.
- Gopaldaswamy, R., P. R. Narayanan and S. Narayanan, (2004) Cloning, overexpression, and characterization of a serine/threonine protein kinase pknI from *Mycobacterium tuberculosis* H37Rv. *Protein Expr Purif* **36**: 82-89.
- Gopaldaswamy, R., S. Narayanan, B. Chen, W. R. Jacobs and Y. Av-Gay, (2009) The serine/threonine protein kinase PknI controls the growth of *Mycobacterium tuberculosis* upon infection. *FEMS Microbiol Lett* **295**: 23-29.
- Grangeasse, C., A. J. Cozzone, J. Deutscher and I. Mijakovic, (2007) Tyrosine phosphorylation: an emerging regulatory device of bacterial physiology. *Trends Biochem Sci* **32**: 86-94.
- Grangeasse, C., P. Doublet and A. J. Cozzone, (2002) Tyrosine phosphorylation of protein kinase Wzc from *Escherichia coli* K12 occurs through a two-step process. *J Biol Chem* **277**: 7127-7135.
- Grangeasse, C., P. Doublet, E. Vaganay, C. Vincent, G. Deleage, B. Duclos and A. J. Cozzone, (1997) Characterization of a bacterial gene encoding an autophosphorylating protein tyrosine kinase. *Gene* **204**: 259-265.

- Greenstein, A. E., J. A. Macgurn, C. E. Baer, A. M. Falick, J. S. Cox and T. Alber, (2007) *M. tuberculosis* Ser/Thr Protein Kinase D Phosphorylates an Anti-Anti-Sigma Factor Homolog. *PLoS Pathog* **3**: e49.
- Grundner, C., L. M. Gay and T. Alber, (2005) *Mycobacterium tuberculosis* serine/threonine kinases PknB, PknD, PknE, and PknF phosphorylate multiple FHA domains. *Protein Sci* **14**: 1918-1921.
- Han, G. and C. C. Zhang, (2001) On the origin of Ser/Thr kinases in a prokaryote. *FEMS Microbiol Lett* **200**: 79-84.
- Hanks, S. K. and T. Hunter, (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *Faseb J* **9**: 576-596.
- Hanks, S. K. and A. M. Quinn, (1991) Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. *Methods Enzymol* **200**: 38-62.
- Hanks, S. K., A. M. Quinn and T. Hunter, (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**: 42-52.
- Hemmer, W., M. McGlone, I. Tsigelny and S. S. Taylor, (1997) Role of the glycine triad in the ATP-binding site of cAMP-dependent protein kinase. *J Biol Chem* **272**: 16946-16954.
- Hernandez, C., A. S. Cetner, J. E. Jordan, S. N. Puangsuwan and J. K. Robinson, (2008) Tuberculosis in the age of biologic therapy. *J Am Acad Dermatol* **59**: 363-380; quiz 382-364.
- Hoch, J. A., (2000) Two-component and phosphorelay signal transduction. *Curr Opin Microbiol* **3**: 165-170.
- Huse, M. and J. Kuriyan, (2002) The conformational plasticity of protein kinases. *Cell* **109**: 275-282.

- Ilan, O., Y. Bloch, G. Frankel, H. Ullrich, K. Geider and I. Rosenshine, (1999) Protein tyrosine kinases in bacterial pathogens are associated with virulence and production of exopolysaccharide. *EMBO J* **18**: 3241-3248.
- Jayakumar, D., W. R. Jacobs, Jr. and S. Narayanan, (2008) Protein kinase E of *Mycobacterium tuberculosis* has a role in the nitric oxide stress response and apoptosis in a human macrophage model of infection. *Cell Microbiol* **10**: 365-374.
- Jin, H. and V. Pancholi, (2006) Identification and biochemical characterization of a eukaryotic-type serine/threonine kinase and its cognate phosphatase in *Streptococcus pyogenes*: their biological functions and substrate identification. *J Mol Biol* **357**: 1351-1372.
- Johnson, L. N., M. E. Noble and D. J. Owen, (1996) Active and inactive protein kinases: structural basis for regulation. *Cell* **85**: 149-158.
- Kang, C. M., D. W. Abbott, S. T. Park, C. C. Dascher, L. C. Cantley and R. N. Husson, (2005) The *Mycobacterium tuberculosis* serine/threonine kinases PknA and PknB: substrate identification and regulation of cell shape. *Genes Dev* **19**: 1692-1704.
- Kang, C. M., K. Vijay and C. W. Price, (1998) Serine kinase activity of a *Bacillus subtilis* switch protein is required to transduce environmental stress signals but not to activate its target PP2C phosphatase. *Mol Microbiol* **30**: 189-196.
- Kannan, N., S. S. Taylor, Y. Zhai, J. C. Venter and G. Manning, (2007) Structural and functional diversity of the microbial kinome. *PLoS Biol* **5**: e17.
- Kaufmann, S. H., (2001) How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol* **1**: 20-30.
- Kirstein, J., D. Zuhlke, U. Gerth, K. Turgay and M. Hecker, (2005) A tyrosine kinase and its activator control the activity of the CtsR heat shock repressor in *B. subtilis*. *EMBO J* **24**: 3435-3445.

- Knighton, D. R., J. H. Zheng, L. F. Ten Eyck, V. A. Ashford, N. H. Xuong, S. S. Taylor and J. M. Sowadski, (1991a) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**: 407-414.
- Knighton, D. R., J. H. Zheng, L. F. Ten Eyck, N. H. Xuong, S. S. Taylor and J. M. Sowadski, (1991b) Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**: 414-420.
- Koul, A., A. Choidas, A. K. Tyagi, K. Drlica, Y. Singh and A. Ullrich, (2001) Serine/threonine protein kinases PknF and PknG of *Mycobacterium tuberculosis*: characterization and localization. *Microbiology* **147**: 2307-2314.
- Krebs, E. G. and E. H. Fischer, (1956) The phosphorylase b to a converting enzyme of rabbit skeletal muscle. *Biochim Biophys Acta* **20**: 150-157.
- Krupa, A. and N. Srinivasan, (2005) Diversity in domain architectures of Ser/Thr kinases and their homologues in prokaryotes. *BMC Genomics* **6**: 129.
- Kumar, P., D. Kumar, A. Parikh, D. Rananaware, M. Gupta, Y. Singh and V. K. Nandicoori, (2009) The *Mycobacterium tuberculosis* protein kinase K modulates activation of transcription from the promoter of mycobacterial monooxygenase operon through phosphorylation of the transcriptional regulator VirS. *J Biol Chem*.
- LaPorte, D. C., (1993) The isocitrate dehydrogenase phosphorylation cycle: regulation and enzymology. *J Cell Biochem* **51**: 14-18.
- LaPorte, D. C. and T. Chung, (1985) A single gene codes for the kinase and phosphatase which regulate isocitrate dehydrogenase. *J Biol Chem* **260**: 15291-15297.
- Leipe, D. D., E. V. Koonin and L. Aravind, (2003) Evolution and classification of P-loop kinases and related proteins. *J Mol Biol* **333**: 781-815.
- Lemaitre, N., W. Sougakoff, C. Truffot-Pernot and V. Jarlier, (1999) Characterization of new mutations in pyrazinamide-resistant strains of *Mycobacterium tuberculosis* and

identification of conserved regions important for the catalytic activity of the pyrazinamidase PncA. *Antimicrob Agents Chemother* **43**: 1761-1763.

Leonard, C. J., L. Aravind and E. V. Koonin, (1998) Novel families of putative protein kinases in bacteria and archaea: evolution of the "eukaryotic" protein kinase superfamily. *Genome Res* **8**: 1038-1047.

Levine, A., F. Vannier, C. Absalon, L. Kuhn, P. Jackson, E. Scrivener, V. Labas, J. Vinh, P. Courtney, J. Garin and S. J. Seror, (2006) Analysis of the dynamic *Bacillus subtilis* Ser/Thr/Tyr phosphoproteome implicated in a wide variety of cellular processes. *Proteomics* **6**: 2157-2173.

Louw, G. E., R. M. Warren, P. R. Donald, M. B. Murray, M. Bosman, P. D. Van Helden, D. B. Young and T. C. Victor, (2006) Frequency and implications of pyrazinamide resistance in managing previously treated tuberculosis patients. *Int J Tuberc Lung Dis* **10**: 802-807.

Macek, B., F. Gnad, B. Soufi, C. Kumar, J. V. Olsen, I. Mijakovic and M. Mann, (2008) Phosphoproteome analysis of *E. coli* reveals evolutionary conservation of bacterial Ser/Thr/Tyr phosphorylation. *Mol Cell Proteomics* **7**: 299-307.

Macek, B., I. Mijakovic, J. V. Olsen, F. Gnad, C. Kumar, P. R. Jensen and M. Mann, (2007) The serine/threonine/tyrosine phosphoproteome of the model bacterium *Bacillus subtilis*. *Mol Cell Proteomics* **6**: 697-707.

Madec, E., A. Laszkiewicz, A. Iwanicki, M. Obuchowski and S. Seror, (2002) Characterization of a membrane-linked Ser/Thr protein kinase in *Bacillus subtilis*, implicated in developmental processes. *Mol Microbiol* **46**: 571-586.

Manning, G., D. B. Whyte, R. Martinez, T. Hunter and S. Sudarsanam, (2002) The protein kinase complement of the human genome. *Science* **298**: 1912-1934.

Marquez, J. A., S. Hasenbein, B. Koch, S. Fieulaine, S. Nessler, R. B. Russell, W. Hengstenberg and K. Scheffzek, (2002) Structure of the full-length HPr kinase/phosphatase from *Staphylococcus xylosus* at 1.95 Å resolution: Mimicking the product/substrate of the phospho transfer reactions. *Proc Natl Acad Sci U S A* **99**: 3458-3463.

- Matsumoto, A., S. K. Hong, H. Ishizuka, S. Horinouchi and T. Beppu, (1994) Phosphorylation of the AfsR protein involved in secondary metabolism in *Streptomyces* species by a eukaryotic-type protein kinase. *Gene* **146**: 47-56.
- Meins, M., P. Jenö, D. Müller, W. J. Richter, J. P. Rosenbusch and B. Erni, (1993) Cysteine phosphorylation of the glucose transporter of *Escherichia coli*. *J Biol Chem* **268**: 11604-11609.
- Mijakovic, I., S. Poncet, G. Boel, A. Maze, S. Gillet, E. Jamet, P. Decottignies, C. Grangeasse, P. Doublet, P. Le Marechal and J. Deutscher, (2003) Transmembrane modulator-dependent bacterial tyrosine kinase activates UDP-glucose dehydrogenases. *EMBO J* **22**: 4709-4718.
- Mijakovic, I., S. Poncet, A. Galinier, V. Monedero, S. Fieulaine, J. Janin, S. Nessler, J. A. Marquez, K. Scheffzek, S. Hasenbein, W. Hengstenberg and J. Deutscher, (2002) Pyrophosphate-producing protein dephosphorylation by HPr kinase/phosphorylase: a relic of early life? *Proc Natl Acad Sci U S A* **99**: 13442-13447.
- Miller, S. P., R. Chen, E. J. Karschnia, C. Romfo, A. Dean and D. C. LaPorte, (2000) Locations of the regulatory sites for isocitrate dehydrogenase kinase/phosphatase. *J Biol Chem* **275**: 833-839.
- Miller, S. P., E. J. Karschnia, T. P. Ikeda and D. C. LaPorte, (1996) Isocitrate dehydrogenase kinase/phosphatase. Kinetic characteristics of the wild-type and two mutant proteins. *J Biol Chem* **271**: 19124-19128.
- Mizuno, T., (1998) His-Asp phosphotransfer signal transduction. *J Biochem* **123**: 555-563.
- Molle, V., R. C. Reynolds, L. J. Alderwick, G. S. Besra, A. J. Cozzone, K. Futterer and L. Kremer, (2008) EmbR2, a structural homologue of EmbR, inhibits the *Mycobacterium tuberculosis* kinase/substrate pair PknH/EmbR. *Biochem J* **410**: 309-317.
- Molle, V., A. K. Brown, G. S. Besra, A. J. Cozzone and L. Kremer, (2006a) The condensing activities of the *Mycobacterium tuberculosis* type II fatty acid synthase are differentially regulated by phosphorylation. *J Biol Chem* **281**: 30094-30103.

- Molle, V., I. Zanella-Cleon, J. P. Robin, S. Mallejac, A. J. Cozzone and M. Becchi, (2006b) Characterization of the phosphorylation sites of *Mycobacterium tuberculosis* serine/threonine protein kinases, PknA, PknD, PknE, and PknH by mass spectrometry. *Proteomics* **6**: 3754-3766.
- Molle, V., D. Soulat, J. M. Jault, C. Grangeasse, A. J. Cozzone and J. F. Prost, (2004) Two FHA domains on an ABC transporter, Rv1747, mediate its phosphorylation by PknF, a Ser/Thr protein kinase from *Mycobacterium tuberculosis*. *FEMS Microbiol Lett* **234**: 215-223.
- Molle, V., C. Girard-Blanc, L. Kremer, P. Doublet, A. J. Cozzone and J. F. Prost, (2003a) Protein PknE, a novel transmembrane eukaryotic-like serine/threonine kinase from *Mycobacterium tuberculosis*. *Biochem Biophys Res Commun* **308**: 820-825.
- Molle, V., L. Kremer, C. Girard-Blanc, G. S. Besra, A. J. Cozzone and J. F. Prost, (2003b) An FHA phosphoprotein recognition domain mediates protein EmbR phosphorylation by PknH, a Ser/Thr protein kinase from *Mycobacterium tuberculosis*. *Biochemistry* **42**: 15300-15309.
- Morona, J. K., J. C. Paton, D. C. Miller and R. Morona, (2000) Tyrosine phosphorylation of CpsD negatively regulates capsular polysaccharide biosynthesis in *streptococcus pneumoniae*. *Mol Microbiol* **35**: 1431-1442.
- Munoz-Dorado, J., S. Inouye and M. Inouye, (1991) A gene encoding a protein serine/threonine kinase is required for normal development of *M. xanthus*, a gram-negative bacterium. *Cell* **67**: 995-1006.
- Narayan, A., P. Sachdeva, K. Sharma, A. K. Saini, A. K. Tyagi and Y. Singh, (2007) Serine threonine protein kinases of mycobacterial genus: phylogeny to function. *Physiol Genomics* **29**: 66-75.
- Nariya, H. and S. Inouye, (2002) Activation of 6-phosphofructokinase via phosphorylation by Pkn4, a protein Ser/Thr kinase of *Myxococcus xanthus*. *Mol Microbiol* **46**: 1353-1366.
- Nariya, H. and S. Inouye, (2003) An effective sporulation of *Myxococcus xanthus* requires glycogen consumption via Pkn4-activated 6-phosphofructokinase. *Mol Microbiol* **49**: 517-528.

- Nariya, H. and S. Inouye, (2005a) Factors that modulate the Pkn4 kinase cascade in *Myxococcus xanthus*. *J Mol Microbiol Biotechnol* **9**: 147-153.
- Nariya, H. and S. Inouye, (2005b) Modulating factors for the Pkn4 kinase cascade in regulating 6-phosphofructokinase in *Myxococcus xanthus*. *Mol Microbiol* **56**: 1314-1328.
- Neu, J. M., S. V. MacMillan, J. R. Nodwell and G. D. Wright, (2002) StoPK-1, a serine/threonine protein kinase from the glycopeptide antibiotic producer *Streptomyces toyocaensis* NRRL 15009, affects oxidative stress response. *Mol Microbiol* **44**: 417-430.
- Nguyen, L. and J. Pieters, (2005) The Trojan horse: survival tactics of pathogenic mycobacteria in macrophages. *Trends Cell Biol* **15**: 269-276.
- Nguyen, L., N. Scherr, J. Gatfield, A. Walburger, J. Pieters and C. J. Thompson, (2007) Antigen 84, an effector of pleiomorphism in *Mycobacterium smegmatis*. *J Bacteriol* **189**: 7896-7910.
- Nixon, B. T., C. W. Ronson and F. M. Ausubel, (1986) Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes ntrB and ntrC. *Proc Natl Acad Sci U S A* **83**: 7850-7854.
- O'Hare, H. M., R. Duran, C. Cervenansky, M. Bellinzoni, A. M. Wehenkel, O. Pritsch, G. Obal, J. Baumgartner, J. Vialaret, K. Johnsson and P. M. Alzari, (2008) Regulation of glutamate metabolism by protein kinases in mycobacteria. *Mol Microbiol* **70**: 1408-1423.
- Ortiz-Lombardia, M., F. Pompeo, B. Boitel and P. M. Alzari, (2003) Crystal structure of the catalytic domain of the PknB serine/threonine kinase from *Mycobacterium tuberculosis*. *J Biol Chem* **278**: 13094-13100.
- Ota, I. M. and A. Varshavsky, (1993) A yeast protein similar to bacterial two-component regulators. *Science* **262**: 566-569.
- Paiment, A., J. Hocking and C. Whitfield, (2002) Impact of phosphorylation of specific residues in the tyrosine autokinase, Wzc, on its activity in assembly of group 1 capsules in *Escherichia coli*. *J Bacteriol* **184**: 6437-6447.

- Papavinasasundaram, K. G., B. Chan, J. H. Chung, M. J. Colston, E. O. Davis and Y. Av-Gay, (2005) Deletion of the *Mycobacterium tuberculosis pknH* gene confers a higher bacillary load during the chronic phase of infection in BALB/c mice. *J Bacteriol* **187**: 5751-5760.
- Pares, S., N. Mouz, Y. Petillot, R. Hakenbeck and O. Dideberg, (1996) X-ray structure of *Streptococcus pneumoniae* PBP2x, a primary penicillin target enzyme. *Nat Struct Biol* **3**: 284-289.
- Parikh, A., S. K. Verma, S. Khan, B. Prakash and V. K. Nandicoori, (2009) PknB-mediated phosphorylation of a novel substrate, N-acetylglucosamine-1-phosphate uridyltransferase, modulates its acetyltransferase activity. *J Mol Biol* **386**: 451-464.
- Pas, H. H. and G. T. Robillard, (1988) Enzyme IIMtl of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system: identification of the activity-linked cysteine on the mannitol carrier. *Biochemistry* **27**: 5515-5519.
- Peirs, P., P. Lefevre, S. Boarbi, X. M. Wang, O. Denis, M. Braibant, K. Pethe, C. Locht, K. Huygen and J. Content, (2005) *Mycobacterium tuberculosis* with disruption in genes encoding the phosphate binding proteins PstS1 and PstS2 is deficient in phosphate uptake and demonstrates reduced in vivo virulence. *Infect Immun* **73**: 1898-1902.
- Peirs, P., B. Parmentier, L. De Wit and J. Content, (2000) The *Mycobacterium bovis* homologous protein of the *Mycobacterium tuberculosis* serine/threonine protein kinase MbK (PknD) is truncated. *FEMS Microbiol Lett* **188**: 135-139.
- Perez, J., R. Garcia, H. Bach, J. H. de Waard, W. R. Jacobs, Jr., Y. Av-Gay, J. Bubis and H. E. Takiff, (2006) *Mycobacterium tuberculosis* transporter MmpL7 is a potential substrate for kinase PknD. *Biochem Biophys Res Commun* **348**: 6-12.
- Pieters, J., (2001) Entry and survival of pathogenic mycobacteria in macrophages. *Microbes Infect* **3**: 249-255.
- Pigiet, V. and R. R. Conley, (1978) Isolation and characterization of phosphothioredoxin from *Escherichia coli*. *J Biol Chem* **253**: 1910-1920.

- Plowman, G. D., S. Sudarsanam, J. Bingham, D. Whyte and T. Hunter, (1999) The protein kinases of *Caenorhabditis elegans*: a model for signal transduction in multicellular organisms. *Proc Natl Acad Sci U S A* **96**: 13603-13610.
- Ponting, C. P., J. Schultz, F. Milpetz and P. Bork, (1999) SMART: identification and annotation of domains from signalling and extracellular protein sequences. *Nucleic Acids Res* **27**: 229-232.
- Popham, D. L. and K. D. Young, (2003) Role of penicillin-binding proteins in bacterial cell morphogenesis. *Curr Opin Microbiol* **6**: 594-599.
- Qamra, R., V. Srinivas and S. C. Mande, (2004) *Mycobacterium tuberculosis* GroEL homologues unusually exist as lower oligomers and retain the ability to suppress aggregation of substrate proteins. *J Mol Biol* **342**: 605-617.
- Rajagopal, L., A. Vo, A. Silvestroni and C. E. Rubens, (2005) Regulation of purine biosynthesis by a eukaryotic-type kinase in *Streptococcus agalactiae*. *Mol Microbiol* **56**: 1329-1346.
- Ramaswamy, S. V., A. G. Amin, S. Goksel, C. E. Stager, S. J. Dou, H. El Sahly, S. L. Moghazeh, B. N. Kreiswirth and J. M. Musser, (2000) Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **44**: 326-336.
- Russell, D. G., (2007) Who puts the tubercle in tuberculosis? *Nat Rev Microbiol* **5**: 39-47.
- Saier, M. H., Jr., L. F. Wu and J. Reizer, (1990) Regulation of bacterial physiological processes by three types of protein phosphorylating systems. *Trends Biochem Sci* **15**: 391-395.
- Sassetti, C. M., D. H. Boyd & E. J. Rubin, (2003) Genes required for mycobacterial growth defined by high density mutagenesis. *Mol Microbiol* **48**: 77-84.
- Scherr, N. (2008) Studies on the Structure and Function of Protein Kinase G, a Virulence Factor of *Mycobacterium tuberculosis*. Thèse, Université de Bâle.

- Schmidt, R., P. Margolis, L. Duncan, R. Coppolecchia, C. P. Moran, Jr. and R. Losick, (1990) Control of developmental transcription factor sigma F by sporulation regulatory proteins SpoIIAA and SpoIIAB in *Bacillus subtilis*. *Proc Natl Acad Sci U S A* **87**: 9221-9225.
- Seeger, M., G. Osorio and C. A. Jerez, (1996) Phosphorylation of GroEL, DnaK and other proteins from *Thiobacillus ferrooxidans* grown under different conditions. *FEMS Microbiol Lett* **138**: 129-134.
- Sharma, K., H. Chandra, P. K. Gupta, M. Pathak, A. Narayan, L. S. Meena, R. C. D'Souza, P. Chopra, S. Ramachandran and Y. Singh, (2004) PknH, a transmembrane Hank's type serine/threonine kinase from *Mycobacterium tuberculosis* is differentially expressed under stress conditions. *FEMS Microbiol Lett* **233**: 107-113.
- Sharma, K., M. Gupta, A. Krupa, N. Srinivasan and Y. Singh, (2006a) EmbR, a regulatory protein with ATPase activity, is a substrate of multiple serine/threonine kinases and phosphatase in *Mycobacterium tuberculosis*. *Febs J* **273**: 2711-2721.
- Sharma, K., M. Gupta, M. Pathak, N. Gupta, A. Koul, S. Sarangi, R. Baweja and Y. Singh, (2006b) Transcriptional control of the mycobacterial *embCAB* operon by PknH through a regulatory protein, EmbR, *in vivo*. *J Bacteriol* **188**: 2936-2944.
- Sherman, M. and A. L. Goldberg, (1994) Heat shock-induced phosphorylation of GroEL alters its binding and dissociation from unfolded proteins. *J Biol Chem* **269**: 31479-31483.
- Shi, L., K. M. Bischoff and P. J. Kennelly, (1999) The *icfG* gene cluster of *Synechocystis sp.* strain PCC 6803 encodes an Rsb/Spo-like protein kinase, protein phosphatase, and two phosphoproteins. *J Bacteriol* **181**: 4761-4767.
- Shi, L., M. Potts and P. J. Kennelly, (1998) The serine, threonine, and/or tyrosine-specific protein kinases and protein phosphatases of prokaryotic organisms: a family portrait. *FEMS Microbiol Rev* **22**: 229-253.
- Shinnick, T. M. and R. C. Good, (1994) Mycobacterial taxonomy. *Eur J Clin Microbiol Infect Dis* **13**: 884-901.

- Shoub, H. L., (1923) A Comparison of the Ziehl-Neelsen and Schulte-Tigges Methods of Staining Tubercle Bacilli. *J Bacteriol* **8**: 121-126.
- Singh, A., Y. Singh, R. Pine, L. Shi, R. Chandra and K. Drlica, (2006) Protein kinase I of *Mycobacterium tuberculosis*: Cellular localization and expression during infection of macrophage-like cells. *Tuberculosis (Edinb)* **86**: 28-33.
- Somoskovi, A., L. M. Parsons and M. Salfinger, (2001) The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in *Mycobacterium tuberculosis*. *Respir Res* **2**: 164-168.
- Soufi, B., F. Gnad, P. R. Jensen, D. Petranovic, M. Mann, I. Mijakovic and B. Macek, (2008) The Ser/Thr/Tyr phosphoproteome of *Lactococcus lactis* IL1403 reveals multiply phosphorylated proteins. *Proteomics* **8**: 3486-3493.
- Soulat, D., J. M. Jault, B. Duclos, C. Geourjon, A. J. Cozzone and C. Grangeasse, (2006) *Staphylococcus aureus* operates protein-tyrosine phosphorylation through a specific mechanism. *J Biol Chem* **281**: 14048-14056.
- Stein, E. A., K. Cho, P. I. Higgs and D. R. Zusman, (2006) Two Ser/Thr protein kinases essential for efficient aggregation and spore morphogenesis in *Myxococcus xanthus*. *Mol Microbiol* **60**: 1414-1431.
- Steinberg, R. A., R. D. Cauthron, M. M. Symcox and H. Shuntoh, (1993) Autoactivation of catalytic (C alpha) subunit of cyclic AMP-dependent protein kinase by phosphorylation of threonine 197. *Mol Cell Biol* **13**: 2332-2341.
- Stuckey, J. A., H. L. Schubert, E. B. Fauman, Z. Y. Zhang, J. E. Dixon and M. A. Saper, (1994) Crystal structure of *Yersinia* protein tyrosine phosphatase at 2.5 Å and the complex with tungstate. *Nature* **370**: 571-575.
- Takayama, K. and J. O. Kilburn, (1989) Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* **33**: 1493-1499.

- Taylor, S. S., (1989) cAMP-dependent protein kinase. Model for an enzyme family. *J Biol Chem* **264**: 8443-8446.
- Taylor, S. S. and E. Radzio-Andzelm, (1994) Three protein kinase structures define a common motif. *Structure* **2**: 345-355.
- Thakur, M., R. Chaba, A. K. Mondal and P. K. Chakraborti, (2008) Interdomain interaction reconstitutes the functionality of PknA, a eukaryotic type Ser/Thr kinase from *Mycobacterium tuberculosis*. *J Biol Chem* **283**: 8023-8033.
- Thakur, M. and P. K. Chakraborti, (2006) GTPase Activity of Mycobacterial FtsZ Is Impaired Due to Its Transphosphorylation by the Eukaryotic-type Ser/Thr Kinase, PknA. *J Biol Chem* **281**: 40107-40113.
- Thakur, M. and P. K. Chakraborti, (2008) Ability of PknA, a mycobacterial eukaryotic-type serine/threonine kinase to transphosphorylate MurD, a ligase involved in the process of peptidoglycan biosynthesis. *Biochem J* **415**:27-33.
- Thomasson, B., J. Link, A. G. Stassinopoulos, N. Burke, L. Plamann and P. L. Hartzell, (2002) MglA, a small GTPase, interacts with a tyrosine kinase to control type IV pili-mediated motility and development of *Myxococcus xanthus*. *Mol Microbiol* **46**: 1399-1413.
- Tinsley, C. R., R. Voulhoux, J. L. Beretti, J. Tommassen and X. Nassif, (2004) Three homologues, including two membrane-bound proteins, of the disulfide oxidoreductase DsbA in *Neisseria meningitidis*: effects on bacterial growth and biogenesis of functional type IV pili. *J Biol Chem* **279**: 27078-27087.
- Tormay, P., A. R. Coates and B. Henderson, (2005) The intercellular signaling activity of the *Mycobacterium tuberculosis* chaperonin 60.1 protein resides in the equatorial domain. *J Biol Chem* **280**: 14272-14277.
- Treuner-Lange, A., M. J. Ward and D. R. Zusman, (2001) Pph1 from *Myxococcus xanthus* is a protein phosphatase involved in vegetative growth and development. *Mol Microbiol* **40**: 126-140.

- Tsai, M. C., S. Chakravarty, G. Zhu, J. Xu, K. Tanaka, C. Koch, J. Tufariello, J. Flynn and J. Chan, (2006) Characterization of the tuberculous granuloma in murine and human lungs: cellular composition and relative tissue oxygen tension. *Cell Microbiol* **8**: 218-232.
- Ulrichs, T. and S. H. Kaufmann, (2006) New insights into the function of granulomas in human tuberculosis. *J Pathol* **208**: 261-269.
- Umeyama, T., P. C. Lee and S. Horinouchi, (2002) Protein serine/threonine kinases in signal transduction for secondary metabolism and morphogenesis in *Streptomyces*. *Appl Microbiol Biotechnol* **59**: 419-425.
- Van Soolingen, D., T. Hoogenboezem, P. E. de Haas, P. W. Hermans, M. A. Koedam, K. S. Teppema, P. J. Brennan, G. S. Besra, F. Portaels, J. Top, L. M. Schouls and J. D. van Embden, (1997) A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex, Canetti: characterization of an exceptional isolate from Africa. *Int J Syst Bacteriol* **47**: 1236-1245.
- Villarino, A., R. Duran, A. Wehenkel, P. Fernandez, P. England, P. Brodin, S. T. Cole, U. Zimny-Arndt, P. R. Jungblut, C. Cervenansky and P. M. Alzari, (2005) Proteomic identification of *M. tuberculosis* protein kinase substrates: PknB recruits GarA, a FHA domain-containing protein, through activation loop-mediated interactions. *J Mol Biol* **350**: 953-963.
- Vincent, C., P. Doublet, C. Grangeasse, E. Vaganay, A. J. Cozzone and B. Duclos, (1999) Cells of *Escherichia coli* contain a protein-tyrosine kinase, Wzc, and a phosphotyrosine-protein phosphatase, Wzb. *J Bacteriol* **181**: 3472-3477.
- Vincent, C., B. Duclos, C. Grangeasse, E. Vaganay, M. Riberty, A. J. Cozzone and P. Doublet, (2000) Relationship between exopolysaccharide production and protein-tyrosine phosphorylation in gram-negative bacteria. *J Mol Biol* **304**: 311-321.
- Walburger, A., A. Koul, G. Ferrari, L. Nguyen, C. Prescianotto-Baschong, K. Huygen, B. Klebl, C. Thompson, G. Bacher and J. Pieters, (2004) Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. *Science* **304**: 1800-1804.

- Walsh, D. A., D. B. Glass and R. D. Mitchell, (1992) Substrate diversity of the cAMP-dependent protein kinase: regulation based upon multiple binding interactions. *Curr Opin Cell Biol* **4**: 241-251.
- Walsh, D. A., J. P. Perkins and E. G. Krebs, (1968) An adenosine 3',5'-monophosphate-dependant protein kinase from rabbit skeletal muscle. *J Biol Chem* **243**: 3763-3765.
- Wehenkel, A., M. Bellinzoni, M. Grana, R. Duran, A. Villarino, P. Fernandez, G. Andre-Leroux, P. England, H. Takiff, C. Cervenansky, S. T. Cole and P. M. Alzari, (2008) Mycobacterial Ser/Thr protein kinases and phosphatases: physiological roles and therapeutic potential. *Biochim Biophys Acta* **1784**: 193-202.
- Wehenkel, A., P. Fernandez, M. Bellinzoni, V. Catherinot, N. Barilone, G. Labesse, M. Jackson and P. M. Alzari, (2006) The structure of PknB in complex with mitoxantrone, an ATP-competitive inhibitor, suggests a mode of protein kinase regulation in mycobacteria. *FEBS Lett* **580**: 3018-3022.
- Wick, M. J., K. R. Wick, H. Chen, H. He, L. Q. Dong, M. J. Quon and F. Liu, (2002) Substitution of the autophosphorylation site Thr516 with a negatively charged residue confers constitutive activity to mouse 3-phosphoinositide-dependent protein kinase-1 in cells. *J Biol Chem* **277**: 16632-16638.
- Wise, A. A. and C. W. Price, (1995) Four additional genes in the sigB operon of *Bacillus subtilis* that control activity of the general stress factor sigma B in response to environmental signals. *J Bacteriol* **177**: 123-133.
- Wu, J., N. Ohta, J. L. Zhao and A. Newton, (1999) A novel bacterial tyrosine kinase essential for cell division and differentiation. *Proc Natl Acad Sci U S A* **96**: 13068-13073.
- Wugeditsch, T., A. Paiment, J. Hocking, J. Drummelsmith, C. Forrester and C. Whitfield, (2001) Phosphorylation of Wzc, a tyrosine autokinase, is essential for assembly of group 1 capsular polysaccharides in *Escherichia coli*. *J Biol Chem* **276**: 2361-2371.

- Xu, B., J. M. English, J. L. Wilsbacher, S. Stippec, E. J. Goldsmith and M. H. Cobb, (2000) WNK1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain II. *J Biol Chem* **275**: 16795-16801.
- Xu, B. E., X. Min, S. Stippec, B. H. Lee, E. J. Goldsmith and M. H. Cobb, (2002) Regulation of WNK1 by an autoinhibitory domain and autophosphorylation. *J Biol Chem* **277**: 48456-48462.
- Yeats, C., R. D. Finn and A. Bateman, (2002) The PASTA domain: a beta-lactam-binding domain. *Trends Biochem Sci* **27**: 438.
- Young, T. A., B. Delagoutte, J. A. Endrizzi, A. M. Falick and T. Alber, (2003) Structure of *Mycobacterium tuberculosis* PknB supports a universal activation mechanism for Ser/Thr protein kinases. *Nat Struct Biol* **10**: 168-174.
- Zhang, C. C., J. Jang, S. Sakr and L. Wang, (2005) Protein phosphorylation on Ser, Thr and Tyr residues in cyanobacteria. *J Mol Microbiol Biotechnol* **9**: 154-166.
- Zhao, X. and J. S. Lam, (2002) WaaP of *Pseudomonas aeruginosa* is a novel eukaryotic type protein-tyrosine kinase as well as a sugar kinase essential for the biosynthesis of core lipopolysaccharide. *J Biol Chem* **277**: 4722-4730.
- Zheng, X., K. G. Papavinasasundaram and Y. Av-Gay, (2007) Novel substrates of *Mycobacterium tuberculosis* PknH Ser/Thr kinase. *Biochem Biophys Res Commun* **355**: 162-168.
- Zimhony, O., J. S. Cox, J. T. Welch, C. Vilcheze and W. R. Jacobs, Jr., (2000) Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FASI) of *Mycobacterium tuberculosis*. *Nat Med* **6**: 1043-1047.
- Zoller, M. J., N. C. Nelson and S. S. Taylor, (1981) Affinity labeling of cAMP-dependent protein kinase with p-fluorosulfonylbenzoyl adenosine. Covalent modification of lysine 71. *J Biol Chem* **256**: 10837-10842.