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Sara Esther Diomande

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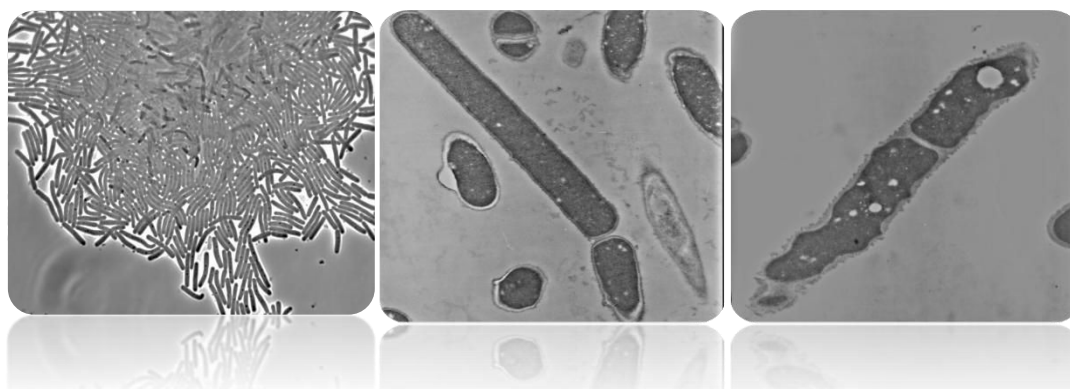
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Sara Esther DIOMANDE



Adaptation au froid de la bactérie pathogène *Bacillus cereus* : étude de mécanismes impliqués et exploitation de la diversité génétique

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Rapporteurs **Dr. Emmanuelle BOUVERET** Directrice de recherche, CNRS, Marseille
 Dr. Pascale SERROR Directrice de Recherche, INRA Jouy-en-Josas

Examineurs **Pr. Catherine DUPORT** Professeur de l'Université d'Avignon
 Pr. Marc FEUILLOLEY Professeur de l'Université de Rouen
 Dr. Vincent SANCHIS Directeur de recherche, INRA Jouy-en Josas

Directeurs de thèse **Dr. Véronique BROUSSOLLE** Chargée de recherche, INRA Avignon
 Dr. Julien BRILLARD Chargé de recherche, INRA Avignon

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INRA PACA Avignon

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(Auteur anonyme)

A ma famille avec toute mon affection

“The measure of intelligence is the ability to change.”

(Albert Einstein)

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Liste des abbréviations

| | |
|----------------------|--|
| A | Adenine |
| ABC | ATP-binding cassette |
| ACP | Acyl carrier protein |
| ADN/DNA | Acide désoxyribonucléique |
| ADNc | ADN complémentaire |
| ADP | Adénosine Di Phosphate |
| AG/FA | Acides gras |
| AGI/UFA | Acides gras insaturés |
| AMP | Adénosine Mono Phosphate |
| Ap | Ampicilline |
| Antéiso | Acides gras ramifiés en antépénultième position (nomenclature Δ) |
| AOAC | Association of Official Analytical Chemists |
| ARN/RNA | Acide ribonucléique |
| ARNm | Acide ribonucléique messenger |
| ARNr | Acide ribonucléique ribosomique |
| ARNt | Acide ribonucléique de transfert |
| ATCC | American Type Culture Collection |
| ATP | Adénosine Tri Phosphate |
| A_w | Activité de l'eau |
| Ba | <i>Bacillus anthracis</i> |
| Bc | <i>Bacillus cereus</i> |
| Bcyt | <i>Bacillus cytotoxicus</i> |
| BCFA | Branched-chain fatty acids |
| Bm | <i>Bacillus mycoides</i> |
| Bp | <i>Bacillus pseudomycoides</i> |
| Bs | <i>Bacillus subtilis</i> |
| Bt | <i>Bacillus thuringensis</i> |
| Bw | <i>Bacillus weihenstephanensis</i> |
| CoA | Coenzyme A |
| C | Cytosine |
| CSP | Cold Shock Protein ou protéine de choc froid |
| DO | Densité optique |

| | |
|-----------------|--|
| Da | Dalton |
| DEAD | Acide aspartique-Acide Glutamique-Alanine-Acide aspartique |
| DTT | Dithiothréitol |
| EDTA | Ethylene Diamine Tetra Acétate |
| EGTA | Ethylene Glycol Tetra-acetic Acid |
| Em | Erythromycine |
| FAME | Fatty acid methyl ester |
| FAS | Fatty acid synthesis |
| G | Guanine |
| GC/MS | chromatographie en phase gazeuse couplée à la spectrométrie de masse |
| HCl | Acide Chlorhydrique |
| HEPES | Acide 4-(2-hydroxyéthyl)-1-pipérazine éthane sulfonique |
| InVs | Institut de veille sanitaire |
| Iso | Acides gras ramifiés en avant dernière position (nomenclature Δ) |
| kb | kilo base |
| Km | Kanamycine |
| LB | Luria Bertani |
| MIDI | Microbial ID inc. |
| NCBI | National center of biotechnology information |
| ORF | Open Reading Frame ou cadre ouvert de lecture |
| P | Phosphate |
| Pb | paire de base |
| PBS | Phosphate Buffer Saline |
| PCR | Polymerase Chain Reaction ou réaction de polymérisation en chaîne |
| PE | Phosphatidyléthanolamine |
| PEP | Phosphoénolpyruvate |
| PG | Phosphatidylglycérol |
| pH | Potentiel hydrogène |
| RACE-PCR | Rapid Amplification of cDNA Ends |
| RPM | Tours par minute |
| RNase | RiboNucléase |
| SDS | Sodium Dodecyl Sulfate |
| T | Thymine |
| TAE | Tris Acetate EDTA |

| | |
|----------------|---|
| TIAC | Toxi Infection Alimentaire Collective |
| Tris | Tris(hydroxymethyl)aminoethane |
| UFC/CFU | Unité formant colonie |
| UV | Ultra Violet |
| X-Gal | 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside |
| Xyl | Xylose |

Avant-propos

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Charles Darwin fut le premier à utiliser le terme « adaptation », en 1859, pour définir les changements survenant chez un organisme visant à augmenter ses chances de survie et de reproduction dans le milieu où il vit. Tout être vivant, quel qu'il soit, est amené à s'adapter, le but étant de survivre notamment à la sélection naturelle. Même si cette notion sous-entend de la compétition, elle met en avant l'extraordinaire capacité qu'on eut les êtres peuplant actuellement notre écosystème à subsister au détriment ou non d'autres espèces éteintes à ce jour, colonisant ainsi un environnement donné.

Parmi les organismes possédant une remarquable capacité d'adaptation, les bactéries ont une bonne place. Ces organismes invisibles à l'œil nu pour la plupart font preuve d'incroyables compétences dans ce domaine. L'ubiquité de certaines en témoigne bien. La pathogénicité d'un microorganisme est une adaptation lui permettant de coloniser un environnement donné, l'hôte. Tous ces aspects donnent un poids à l'adaptation et en particulier à celle associée aux pathogènes.

Bacillus cereus, bacille majoritairement présent dans les sols mais retrouvé partout dans l'environnement est un exemple de bactérie ubiquitaire. Cette bactérie est aujourd'hui reconnue comme un pathogène émergent et en 2012 a été répertorié comme 2^e cause de toxi-infection collective (TIAC) en France par l'institut national de veille sanitaire (InVS, 2014). Néanmoins, ce pathogène est considéré comme très peu dangereux et ce à cause de la haute dose minimale infectante et à cause de la faible proportion de cas de mortalité associés à l'infection à *B. cereus* (Tétart Gilles, 2009) même si au regard de ces capacités d'adaptation (Stenfors Arnesen et al., 2008b), cette bactérie fait de plus en plus l'objet de surveillance particulière (InVS, 2014). De plus, certaines souches de *B. cereus* ont été décrites comme possédant des aptitudes de psychrotolérance, c'est-à-dire de tolérance à des températures assez basses et très proches des températures de réfrigération des aliments. Cela suggère pour ces souches une meilleure persistance dans les aliments conservés au froid par rapport aux souches mésophiles. Certaines de ces souches psychrotolérantes, sont aussi capables d'induire des toxi-infections (Thorsen et al., 2006b, Thorsen et al., 2009). Développer des moyens de lutte afin de maîtriser le risque microbiologique associé à *B. cereus* implique la compréhension des mécanismes coordonnant l'adaptation aux températures froides.

Les recherches menées au sein de l'équipe Microbiologie et sécurité de l'UMR Sécurité et Qualité des Produits d'Origine Végétale sont centrées sur l'étude des mécanismes d'adaptations de *B. cereus* à différentes conditions environnementales incluant les basses températures.

Cette étude s'inscrit dans la démarche d'une meilleure compréhension des mécanismes moléculaires d'adaptation de *B. cereus* aux basses températures. Elle permettra à long terme une meilleure maîtrise des risques associés au développement et la persistance de ce germe dans les aliments réfrigérés.

Chapitre 1 :

Etude bibliographique

Chapitre 1 : Etude bibliographique

I) *Bacillus cereus*

1) *B. cereus*, généralités

B. *cereus* est un bacille à coloration Gram positive, aérobic-anaérobic facultatif, sporulé (Figure 1). Cette bactérie a été isolée pour la première fois au Royaume-Uni en 1887 suite à un prélèvement de l'air d'une étable (Frankland, 1887). Le nom de *Bacillus* fut attribué à cette bactérie à cause de la forme en « petite tige » de ses cellules végétatives, « small rod » en anglais, et *cereus* à cause de l'aspect cireux des colonies sur boîte de Pétri. Cette première souche isolée est aujourd'hui la souche type de l'espèce, la souche ATCC (American Type Culture Collection) 14579.

Il faut attendre le Docteur norvégien Steinar Hauge pour voir émerger les recherches sur *B. cereus* (Hauge, 1955). En effet, celui-ci fut le premier à prouver que *B. cereus* pouvait causer des infections alimentaires après avoir ingéré une sauce vanille stérile qu'il avait lui-même artificiellement contaminé par une souche de *B. cereus* isolée d'une épidémie de toxoinfection alimentaire (Hauge, 1955). Il développa 16 heures après ingestion des symptômes de diarrhées qui ont été attribués à *B. cereus*. Un peu plus tard en 1974, il fut démontré pour la première fois que ce bacille pouvait aussi être responsable d'empoisonnement alimentaire de type émétique (Mortimer and McCann, 1974).

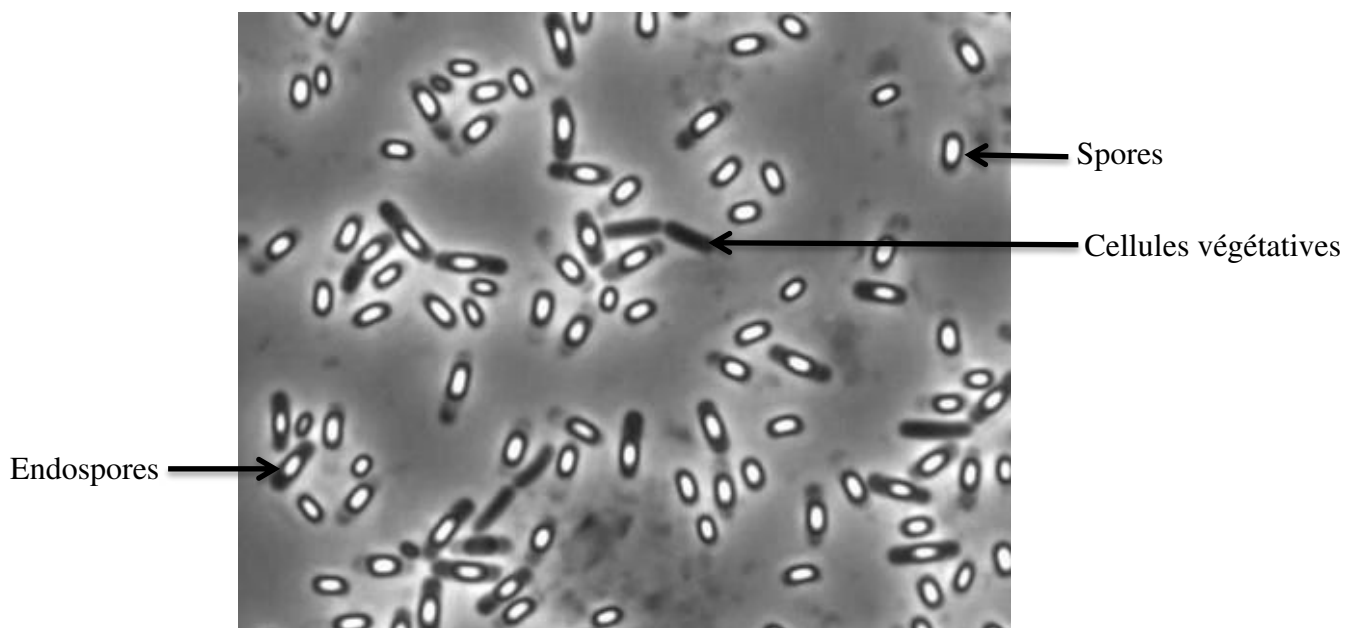


Figure 1 : Cellules végétatives (corps opaques), spores (corps réfringents) et endospores (spores en devenir) de *Bacillus cereus* ATCC 14579 (INRA, Avignon).

2) *B. cereus*, un pathogène alimentaire non négligeable

B. cereus est aujourd'hui bien reconnu pour sa capacité à causer des toxi-infections alimentaires. En 2012, ce pathogène a été identifié comme le 2^e agent responsable de toxi-infection alimentaire collective (TIAC) en France, étant incriminé dans 21 % des foyers de TIAC déclarés, juste après *Staphylococcus aureus* (InVS, 2014). Ce pathogène représente aussi un danger alimentaire rencontré un peu partout dans le monde (Bennett et al., 2013a, Zhou et al., 2014, Ombui et al., 2001, Eglezos et al., 2010). Pourtant les cas de TIAC à *B. cereus* ont longtemps été sous-estimés car les symptômes associés à ce pathogène sont généralement bénins, à durée limitée, ne nécessitent pas d'intervention médicale et ne sont pas à déclaration obligatoire contrairement à d'autres pathogènes alimentaires tels que *Salmonella enteritidis* et *Listeria monocytogenes*. De plus, la dose minimale infectante (DMI) de *B. cereus* est estimée à 10⁵ UFC/ml (Granum et al., 1995). En France, les cas de TIAC à *B. cereus* ont longtemps été considérés comme marginaux pour cette raison, cependant depuis quelques années ce pathogène est sous surveillance. En effet, les cas de TIAC incriminant *B. cereus* était de 0,7 en 1999 contre 21% en 2012 (InVS, 2014, Schmidt, 2003). Beaucoup d'aliments sont associés aux cas de TIAC à *B. cereus*, parmi lesquels le riz, le lait, les viandes, les légumes, les pommes de terres, les haricots noirs fermentés et même de l'eau contaminée... (Delbrassinne et al., 2012, Choi et al., 2011, Ombui et al., 2001, Gaulin et al., 2002, Zhou et al., 2014, Decousser et al., 2013).

3) *B. cereus*, ses toxines

Les diarrhées causées par *B. cereus* sont des infections dues aux formes végétatives de la bactérie qui produisent des enterotoxines dans l'intestin de l'homme. Certaines enterotoxines sont responsables de la formation de pores transmembranaires chez les cellules hôtes entraînant une augmentation de la perméabilité membranaire (Haug et al., 2010, Fagerlund et al., 2008, Beecher et al., 2000). Les plus étudiées sont l'hémolysine BL (Hbl), l'entérotoxine non-hémolytique Nhe et la cytotoxine K (CytK). L'entérotoxine Hbl est composée de deux composants lytiques L2 et L1 et de la protéine de liaison B, ces protéines sont codées respectivement par *hblC*, *hblD* et *hblA* (Heinrichs et al., 1993, Ryan et al., 1997). Un quatrième composant HblB' codé par le gène *hblB'* a été mis en évidence chez la souche ATCC 14579, mais sa fonction est encore inconnue (Clair et al., 2010). Hbl est une

hémolysine car elle ne possède pas uniquement des propriétés entérotoxiques mais aussi des propriétés hémolytiques.

L'enterotoxine Nhe est également sous la forme d'un complexe protéique tripartite non-hémolytique composé de NheA, NheB et NheC codés par l'opéron *nheABC* (Lindback et al., 2004).

L'enterotoxine CytK est une protéine fonctionnant seule et codée par *cytK*. Deux variants de cette protéine existent : CytK1 et CytK2.

Le rôle des différentes enterotoxines dans l'apparition des diarrhées varie selon les souches et implique l'interaction entre plusieurs toxines (Stenfors Arnesen et al., 2008b). De plus, des études ont démontré que l'action de ces enterotoxines ne se limite pas aux cellules gastro-intestinales mais à d'autres lignées cellulaires (Jessberger et al., 2014). La production de ces toxines est influencée par plusieurs facteurs tels que la composition du milieu, le pH, l'aération et la concentration de certains glucides (McKillip, 2000). Les gènes codant pour ces toxines font partie du régulon de virulence contrôlé par le régulateur transcriptionnel pléiotrope PlcR (Gohar et al., 2008)

En plus de ces enterotoxines *B. cereus* produit d'autres protéines moins caractérisées telles que l'entérotoxine FM (entFM ou cwpFM) (Asano et al., 1997) ainsi que d'autres hémolysines : la céréolysine O (Kreft et al., 1983, Brillard and Lereclus, 2007b), l'hémolysine II (Baida et al., 1999), l'hémolysine III (Baida and Kuzmin, 1996) et la céréolysine AB (Gilmore et al., 1989). D'autres protéines de virulence sont aussi sécrétées par *B. cereus* qui lui permettent de s'échapper aux macrophages du système immunitaire de l'hôte comme des métalloprotéases (Guillemet et al., 2010) ou IIsA qui est une protéine de surface nécessaire à l'acquisition du fer (Daou et al., 2009).

Le syndrome émétique de *B. cereus* est causé par un dodécapeptide nommé céréulide ou toxine émétique (Agata et al., 1994). Ce peptide cyclique est extrêmement stable et résiste à de hautes températures (121°C, 15 min), à une large gamme de pH ainsi qu'aux enzymes protéolytiques du tractus gastro-intestinal (Ehling-Schulz et al., 2004, Shinagawa et al., 1996). Ainsi, lorsque le céréulide est produite dans l'aliment par la bactérie, cette toxine peut persister même après la mort de la bactérie et causer un empoisonnement émétique au consommateur après ingestion de l'aliment contaminé. De nombreux cas ont été décrits dans le passé (Kim et al., 2010, Kamga Wambo et al., 2011, Delbrassinne et al., 2012, Dierick et al., 2005b, Zhou et al., 2014). La dose de céréulide induisant des syndromes émétiques est très peu connue, elle a cependant été estimée lors d'étude réalisées sur des animaux tels que la

musaraigne *Suncus murinus* et le singe rhesus entre 8 et 10 µg/kg de masse corporelle (Shinagawa et al., 1995, Agata et al., 1995). Cependant une étude montre que cette toxine peut avoir un effet inhibiteur sur les cellules immunitaires « natural killer » (NK) de l'homme a des doses plus basses (Paananen et al., 2002).

Agata *et al.* a démontré que les aliments avaient une influence sur la production de céréulide (Tableau 1). Le riz et les aliments liquides agités semblent être les meilleurs substrats pour la production de cette toxine.

Tableau 1 : Influence de l'aliment sur la production de céréulide (Agata et al, 2002) (Agata et

| Aliment | Titre de toxine (ng/g) | Dénombrement (UFC/g) environ |
|--|------------------------|------------------------------|
| Lait (agité) | 640 | 4×10^8 |
| Riz frit | 320 | 3×10^8 |
| Riz cuit à l'eau | | 3×10^8 |
| Riz cuit à l'eau | | 3×10^8 |
| Lait de soja (agité) | | 3×10^8 |
| Spaghetti | 160 | 7×10^8 |
| Purée de pommes de terre | | 5×10^8 |
| Nouilles asiatiques | 80 | 2×10^8 |
| Gruau de riz | 40 | 1×10^8 |
| Pain | 20 | 5×10^8 |
| Gâteau | | 4×10^8 |
| Lait (stationnaire) | < 5 à 10 | 9×10^7 |
| Lait de soja (stationnaire) | | 8×10^7 |
| Œuf et dérivés | | 4×10^7 |
| Spaghetti au ketchup | < 5 | 2×10^5 |
| « Salade de pomme de terre » (purée avec mayonnaise) | | 2×10^6 |
| Viande et ses produits | | 3×10^6 |

En plus de ces syndromes, *B. cereus* peut causer des infections non gastro-intestinales telles que des parodontites (inflammation des tissus de soutien de l'organe dentaire), des endophtalmites fulminantes (inflammation des tissus internes de l'œil), des méningites et autres infections rencontrées chez les personnes immuno-déprimées (Bottone, 2010). Ces

infections cliniques sont problématiques car *B. cereus* est résistant aux antibiotiques tels que les pénicillines et les céphalosporines fréquemment utilisés comme traitement pour ce type d'infection (Garcia Arribas et al., 1988, Katsuya et al., 2009).

4) *B. cereus*, un germe d'altération

Comme indiqué précédemment, *B. cereus* est très présent dans les sols, il est capable de contaminer les aliments issus du sol destinés à la consommation humaine ou animale. Cette présence dans les aliments peut conduire à l'altération de ceux-ci, en réduisant leur demi-vie. Ceci conduit donc à d'énormes pertes pour les industries agroalimentaires en particulier dans la filière laitière qui est la plus touchée (Vithanage et al., 2014, Ternstrom et al., 1993, Arslan et al., 2014, Lucking et al., 2013). Ceci peut s'expliquer par le fait que les aliments utilisés pour le bétail, généralement des végétaux, sont très fréquemment contaminés par *B. cereus*, les fèces du bétail le sont aussi par conséquent, en plus de l'environnement de la ferme incluant les produits laitiers obtenus à partir de ce bétail le sont tout autant (Magnusson et al., 2007). D'autres filières sont néanmoins aussi impactées, telles la filière avicole (Techer et al., 2014) ou la filière des plats cuisinés (Choma et al., 2000, Guinebretiere et al., 2003).

Les spores de *B. cereus* lui confèrent la capacité de persister dans les aliments durant les procédés de transformations (Carlin, 2011). De plus, les spores et les cellules végétatives de la bactérie sont capables de former des biofilms en s'attachant à des équipements de transformation, à la surface de produits d'emballage et même à la surface des aliments (Elhariry, 2011, Altman et al., 2009, Kreske et al., 2006). Ces biofilms sont des structures multicellulaires renforcées par une matrice d'exopolymères, cette configuration rend difficile l'éradication des microorganismes constituant ce biofilm, par l'utilisation de produits désinfectants classiques (Shaheen et al., 2010, Sundberg et al., 2011, Kreske et al., 2006).

Les souches de *B. cereus* isolées d'altération d'aliments sont généralement des souches produisant des enterotoxines cependant certaines peuvent être des souches émétiques (Thorsen et al., 2009, Arslan et al., 2014)

5) *B. cereus sensu lato*

B. cereus sensu stricto (*ss*) est un membre de *B. cereus sensu lato* (*sl*). Sept espèces génétiquement très proches constituent le groupe *B. cereus sl* : *Bacillus cereus*, *Bacillus*

anthracis, *Bacillus thuringiensis*, *Bacillus mycooides*, *Bacillus pseudomycooides*, *Bacillus cytotoxicus*, and *Bacillus weihenstephanensis*. Comme *B. cereus* ss, ce sont tous des bacilles à Gram positive, aérobie-anaérobie facultatifs et sporulés (Guinebretiere et al., 2013c, Ceuppens et al., 2013, Guinebretiere et al., 2008b)

B. anthracis est l'agent responsable de la maladie du charbon, une maladie sévère qui affecte premièrement les mammifères herbivores (Mock and Fouet, 2001) mais il représente aussi un dangereux pathogène humain (Kalamas, 2004). Ces souches virulentes se caractérisent par la présence d'une capsule constituée d'un polymère d'acide D-glutamique et la production d'exotoxines dont les gènes sont portés par les plasmides pXO1 et pXO2 (Koehler, 2009).

B. thuringiensis se distingue des autres espèces par la formation durant la sporulation d'un corps d'inclusion parasporal. Ce corps d'inclusion est constitué de protéines, les protéines Cry, des endotoxines qui possèdent des propriétés insecticides (Bravo et al., 2011).

B. mycooides est caractérisé par la formation de colonies rhizoïdes ou mycoïdes sur milieu gélosé et par une absence de mobilité (Paul Vos, 2011).

B. pseudomycooides est phénotypiquement identique à *B. mycooides* mais se distingue par comparaison de l'ADN et de la composition en acides gras (Nakamura, 1998). *Bacillus cytotoxicus* est thermotolérant et très souvent associé aux intoxications alimentaires (Guinebretiere et al., 2013c, Lapidus et al., 2008a).

B. weihenstephanensis est une espèce psychrotolérante pouvant se développer de 5°C à 37°C (Guinebretiere et al., 2008b). Cette espèce a été caractérisée par la présence de séquences spécifiques à l'intérieur des gènes de l'ARN 16S et d'une cold-shock protein majeure *cspA* (Lechner et al., 1998, Francis et al., 1998, Pruss et al., 1999). Elle est capable de contaminer les produits laitiers et réfrigérés (Larsen and Jorgensen, 1997, Meer et al., 1991)

B. cereus est comme précédemment expliqué, un germe responsable de toxi-infections alimentaires ou d'infections locales et systémiques chez l'homme.

Comme toutes ces informations l'indiquent la différenciation des souches de *B. cereus* s'appuie donc principalement sur des propriétés généralement phénotypiques.

Afin d'éclaircir la classification existante, Guinebretière *et al.* ont réalisé une étude sur un panel de 425 souches couvrant la diversité phénotypique et génotypique de *B. cereus* s (Guinebretiere et al., 2008b).

Plusieurs techniques d'analyses ont été utilisées et associées pour cette étude phylogénétique : les profils fAFLP (Fluorescent Amplified Fragment Length Polymorphism), la phylogénie des gènes ribosomiaux et d'un gène de ménage approprié le gène *panC* (Guinebretiere et al.,

2008b). Cette étude a permis d'identifier 7 groupes phylogénétiques possédant chacun une gamme de température de croissance bien définie (Figure 2). Ces 7 groupes représentent quatre thermotypes principaux :

- 3 groupes mésophiles : les groupe I, III et IV
- 1 groupe thermotolérant : le groupe VI
- 2 groupes psychrotolérants : les groupe II et VI
- 1 groupe intermédiaire entre les groupes mésophiles et les groupes psychrotolérants : le groupe V.

Comme il est possible de le voir, on peut retrouver toutes les souches d'une même espèce réparties dans un seul et même groupe comme c'est le cas de *B. cytotoxicus* (groupe VII), *B. pseudomycoïdes* (groupe I) et *B. weihenstephanensis* (groupe VI) et *B. anthracis* (groupe III). Cependant les souches de l'espèce *B. cereus*, *B. thuringensis* et *B. mycoïdes* sont réparties dans plusieurs groupes. Cela suggère donc que, par exemple, les souches de *B. cereus ss* du groupe III seraient phylogénétiquement plus proches des souches de *B. anthracis* appartenant au même groupe que des souches de *B. cereus ss* du groupe IV. Cette étude a montré comment l'adaptation à de nouveaux environnements, par la modification des températures limites tolérées, a permis de modéliser historiquement une diversification écologique de *B. cereus sl* (Guinebretiere et al., 2008b).

La température représente donc un point clé dans la diversification de *B. cereus sl*.

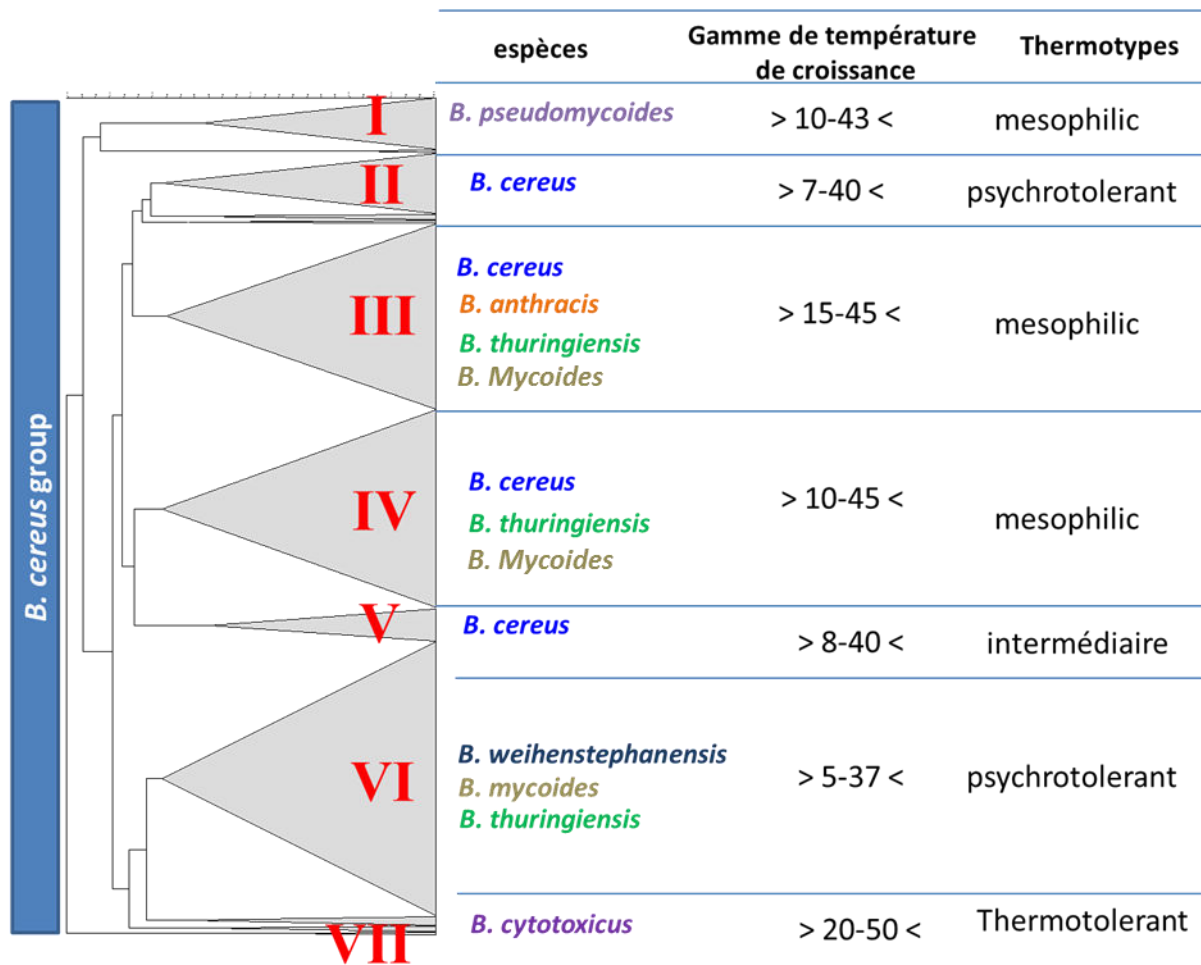


Figure 2 : Diversité génétique et écologique de *B. cereus sl* (Guinebretière *et al.*, 2008)

Par conséquent, les différences de capacité d'adaptation aux changements environnementaux des souches de *B. cereus sl* semblent être un excellent moyen de les discriminer les unes des autres.

II) Rôle de la membrane dans l'adaptation du genre *Bacillus* à l'environnement

Dans la littérature scientifique, il est souvent exposé que l'adaptation du genre *Bacillus* aux divers environnements et notamment au froid est en relation avec leur composition membranaire. La composition membranaire et plus particulièrement la composition en acides gras membranaires des souches est d'ailleurs systématiquement utilisée en taxonomie pour identifier de nouvelles souches, sous-espèces ou espèces. Cette partie de l'étude a pour but de vous présenter les données bibliographiques mettant en évidence le rôle des acides gras membranaires dans l'adaptation des souches du groupe *Bacillus* aux différents environnementaux.

Je vous propose donc de vous présenter ces données bibliographiques sous la forme d'un article de synthèse car aucune synthèse récente à ce propos n'existe à ma connaissance.

Role of fatty acids in *Bacillus* strains adaptation to environment

Sara Esther Diomandé^{1,2}, Christophe Nguyen-the^{1,2}, Marie-Hélène Guinebretière^{1,2},
Véronique Broussolle^{1,2} and Julien Brillard^{1,2} #.

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¹ INRA, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France

² Université d'Avignon, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France.

³UMR 1333 DGIMI, INRA-Université Montpellier, 34095 Montpellier cedex 5

Corresponding author: julien.brillard@univ-montp2.fr

Les références de chaque article sont toutes classées dans la partie Références du manuscrit (page 188)

Abstract

The genus *Bacillus* is a large and diverse bacterial genus widely distributed in environment which belongs to different niches. This genus also includes pathogens and among them pathogens responsible for foodborne illness (FBI) outbreaks. The study of the *Bacillus* diversity often includes an analysis of the fatty acid (FA) composition. In this review, we highlight that even if *Bacillus* strains are characterized by a common FA signature, some key particularities in the FA composition among this genus allow to discriminate the *Bacillus* species. However, *Bacillus* species are able to modify their FA pattern for adaptation to a wide range of environmental changes including growth medium changes, temperature changes, food processing conditions, pH... As the other Gram positive bacteria, *Bacillus* strains display a well-defined FA synthesis II system equilibrated with FA degradation pathway in order to efficiently respond to the cell needs. As endogenous FAs, exogenous FAs may impact the survival of *Bacillus* vegetative cells and spores properties in a given environment. Some of these exogenous FAs may represent a powerful mean to preserve food from occurrence of *Bacillus* pathogenic strains responsible for FBI.

Keywords:

Bacillus genus, adaptation, FASII, FA degradation, exogenous FAs.

Introduction

Most of the vital bacterial cell functions are attributed to the plasma membrane (Parsons and Rock, 2013). The main functions of the membrane remains its ability to form a permeable barrier, pump essential metabolites and macromolecules into the cell and prevent the entry of undesirable solutes of the external environment (Ramos et al., 2001, Weber and de Bont, 1996). In order to adapt to a wide range of environments, bacteria have the ability to control the biophysical properties of their membrane (Zhang and Rock, 2008, Murínová et al., 2014) including the membrane fluidity necessary for growth and survival of bacteria in their environment (Esser and Souza, 1974, de Sarrau et al., 2012c). Cell membranes are mainly composed of proteins, RNAs and phospholipids (Bishop et al., 1967a, Beaman et al., 1974). Glycerophospholipids, glycerol-based phospholipids, represent about 90% of macromolecules in bacteria (Neidhardt, 1996). They are mainly located on cell membranes and represent the main pool of FAs in microorganisms. In addition, FAs are important sources of metabolic energy and important effector molecules that regulate metabolism.

FA composition of bacterial cells varies depending on species and has thus been used as biomarkers in taxonomy for years (Cherniavskaia and Vasiurenko, 1983, Vasiurenko et al., 1984, Guinebretiere et al., 2013a). Moreover, FAs composition of cell membrane varies depending on environmental conditions as it plays a leading role in bacterial adaptation to environmental changes (Yano et al., 1998, Sinensky, 1971, de Sarrau et al., 2012c). Exogenous FAs were shown to influence the growth ability of *Bacillus* cells. For example, in *B. cereus* these exogenous FAs may impair (Lee et al., 2002b) or improve the growth (de Sarrau et al., 2013d) depending on environmental conditions. In the last decade, the regulation of genes involved in metabolism and transport of FAs has been described in bacteria (Schujman et al., 2003, Pech-Canul et al., 2011). Only few regulators have been identified (Dirusso and Black, 2004a, Schujman et al., 2003).

The genus *Bacillus* is the largest, most diverse and most prominent aerobic endospore-forming bacteria (Fritze, 2004). Presently, this genus contains about 241 species and 5 subspecies (Euzeby, 2010). *Bacillus* strains are widely distributed in the environment belonging to different niches and including strains with economic and health interest. For instance, species of the genus *Bacillus* can have important roles in industrial processes (de la Fuente-Salcido et al., 2013, Liu et al., 2014, Pasvolsky et al., 2014) and some strains are also human pathogens (Bartoszewicz et al., 2013, Lamanna and Jones, 1963).

This review focuses on the role of FA in *Bacillus* adaptation to their close environment. Here, we first describe the FA composition of *Bacillus* species, by identifying common and specific elements of their FA pattern in relation with their ecological niches. The versatility of the FA pattern for adaptation to changing environment is secondly discussed. Then, the *Bacillus* specific FA biosynthesis and degradation are described and discussed. Finally, the impact of exogenous FA on *Bacillus* species growth ability is discussed.

1- Fatty acids composition in *Bacillus*

1-1 Description of FA profiles in *Bacillus*

As for other Gram positive bacteria, *Bacillus* species have 3 main groups of FAs: branched-chain FA, straight-chain FA and complex FA types (such as cyclic, hydroxyl or epoxy FAs) (Harris, 1996). The genus *Bacillus* is characterized by a homogeneity in the FA composition in opposition to the genus *Micrococcus*, *Clostridium* and *Corynebacterium* (Moss and Lewis, 1967, Harris, 1996). Until now, there is no *Bacillus* strain described for having only branched-chain or straight-chain FA.

Another specificity of this genus is that saturated FA like C_{14:0} or C_{16:0} which are encountered in the majority of microorganisms are generally minor constituents in the genus *Bacillus* (Kaneda, 1977).

1-1-1 Branched-chain FAs

The particularity qualifying the *Bacillus* genus is the abundance of branched-chain FA (Kaneda, 1977, Kämpfer, 1994), with a predominance of branched chain iso and anteiso FAs containing 12-17 carbons (Berkeley, 2002). Branched-chain FAs also includes ω -alicyclic FA with or without modifications like unsaturation and hydroxylation. Branched-chain FAs represent up to 98 % of total fatty acid depending on the species (Kaneda, 1969). More precisely, iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0}, C_{16:0}, iso-C_{17:0}, anteiso-C_{17:0} represent the major FAs typically found in *Bacillus* species (Kämpfer, 1994, Song et al., 2000). A particular group of species among the genus *Bacillus*, *Bacillus cereus sensu lato* is characterized by a lower amounts of anteiso-C_{15:0} and the presence of some particular FAs containing 12 and 13 carbons (isoC_{12:0}, iso-C_{13:0}, anteiso-C_{13:0}) (Song et al., 2000).

1-1-2 Unsaturated FAs

Unsaturated fatty acids (UFAs) proportion varies among the genus *Bacillus*. It was shown to vary from 0 to 28 % of total FAs in optimal growth condition according to Kaneda (Kaneda, 1977). In *Bacillus* strains, UFAs consist almost exclusively in mono-unsaturated FAs at optimal growth temperature (Kaneda, 1977). The most common mono-unsaturated FAs generally encountered in living organisms are $\Delta 9$ -isomers. But, most of the *Bacillus* strains also displayed $\Delta 5$, $\Delta 8$ and $\Delta 10$ -isomers (Kaneda, 1977, Brillard et al., 2010c, de Sarrau et al., 2012c). These bacteria use an aerobic mechanism for the biosynthesis of UFAs in contrast to species from other genus like *Clostridium butyricum*, *Lactobacillus arabinosus*, *Escherichia coli* that are alternatively or additionally using an anaerobic mechanism of UFAs biosynthesis (Scheuerbrandt and Bloch, 1962).

1-1-3 complex FAs

Several *Bacillus* species display unusual FAs. These complex FAs are generally hydroxyl and epoxy FAs. Some *Bacillus* strains also display an unusual type of FA, the hydroxy-FA (Romano et al., 2013, Mondol et al., 2013, Baidara et al., 2013). Hydroxy-FA, unsaturated or not, are linked to polypeptides and form antimicrobial agents (Baidara et al., 2013, Mondol et al., 2013). Epoxy-FAs, consisting of FAs with one or two epoxy group(s), are bioactive molecules that are found among the *Bacillus* genus species (Hou, 2008, Celik et al., 2005).

1-2 FA profiles vary among *Bacillus* species

The *Bacillus* genus is a large genus in which the number of novel strains characterized is increasing (Zhao et al., 2014, Kosowski et al., 2014, Choi and Cha, 2014). Classification of strains in the *Bacillus* genus uses the FAs as biomarkers and tool for taxonomy. Several observations justify this particularity and they are discussed below.

1-2-1 discrimination on UFAs proportion

According to Kaneda *et al*, the *Bacillus* genus strains can be divided into 3 groups depending on their UFAs proportions (Kaneda, 1977).

First of all, species displaying a very low or insignificant proportion of UFAs, from 0 to less than 3% of total FA. This group includes mesophilic and thermophilic strains like *B. subtilis*, *B. megaterium*, *B. stearothermophilus*, *B. acidocaldarius* (see Figure 3). Further studies confirmed that at optimal temperature *B. subtilis* strains displays nearly zero UFAs (Bishop et al., 1967a, Grau and de Mendoza, 1993).

Secondly, strains displaying a small proportion of UFAs, from 7-12% of total FAs. This group was shown to include strains of *B. cereus* group. However, recent studies on *B. cereus* FA composition showed that UFAs proportion may reach higher values (22-28% of total FAs) (Pichinoty, 1984, de Sarrau et al., 2012c). This observation may be due to many factors such as the detection of new UFAs during this 40 last years, or a better sensitivity of analytical tools. Also, some bacteria of this group, such as *B. weihenstephanensis* and *B. mycoides*, psychrotolerant strains of *Bacillus cereus sensu lato* have been described after the Kaneda classification. Moreover, the *Bacillus cereus sensu lato* group is today know to consist of thermotolerant (recently identified, (Guinebretiere et al., 2013a)), mesophilic and psychrotolerant strains.

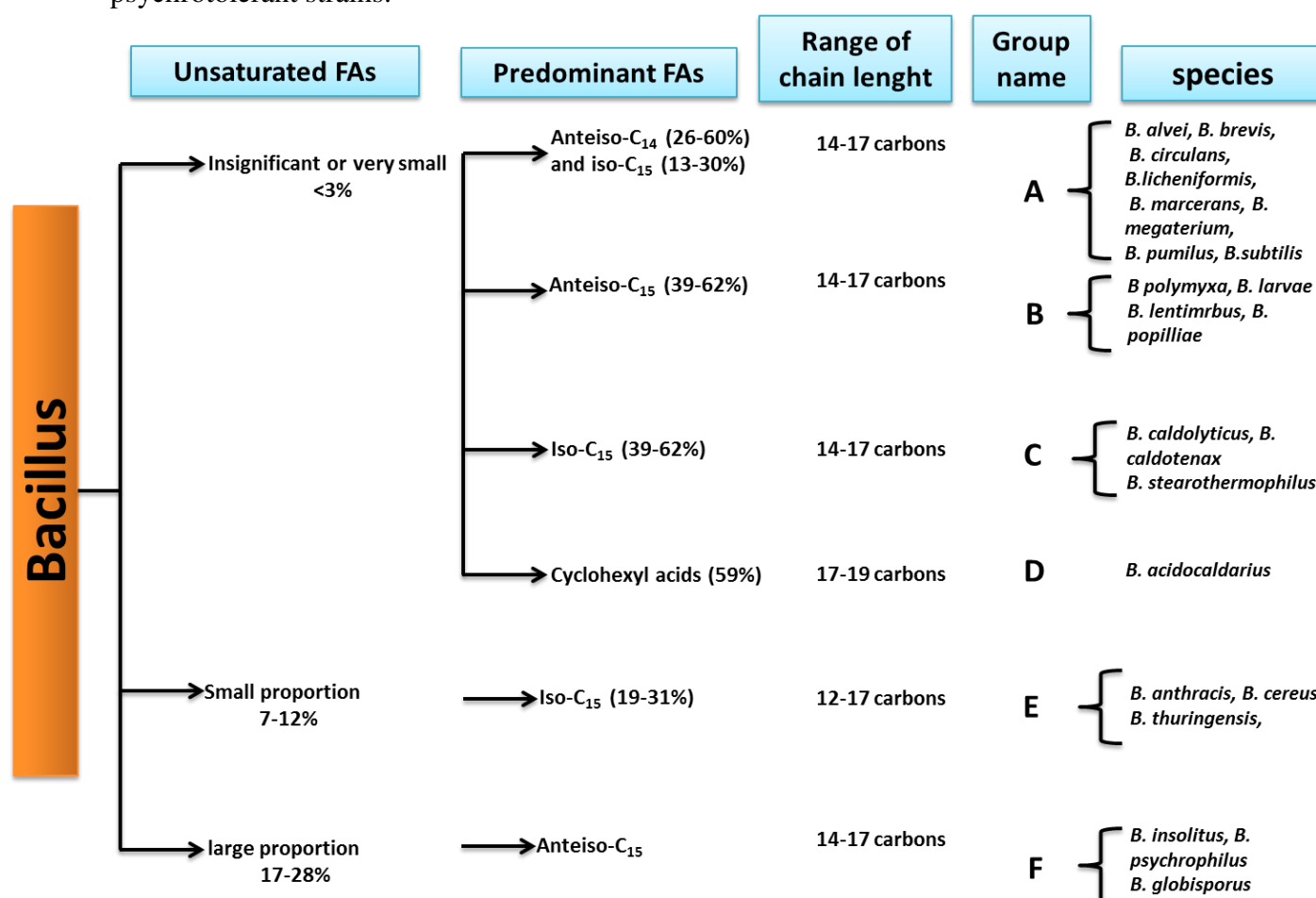


Figure 3: *Bacillus* species classification based on their fatty acids patterns, from Kaneda, 1977

The third group consists only in psychrotolerant species of the genus, described to present higher UFAs proportions (17-28%). Studies realized on three psychrotolerant species revealed that they displayed higher proportion of UFA when compared to other strains, i.e in *Bacillus globisporus* 26.1%, *Bacillus insolitus* 25.1% , *Bacillus psychrophilus* 18.4% of total FAs (Kaneda et al., 1983). Moreover, these UFAs were in majority $\Delta 5$ -isomers meaning that the FAs were unsaturated at the position $\Delta 5$ in the carbon chain (Kaneda, 1971).

In the *Clostridium* genus, it was also shown that the mesophilic and psychrophilic strains were characterized by displaying a higher percentage of UFAs than thermophilic strains. (Chan et al., 1971).

Recent studies indicated that proportion of specific FAs, known for high or low temperature adaptation, including UFAs, contributed to discriminate different ecotypes (a taxonomic subgroups that develops or survives given environmental conditions) among the *B. subtilis* and *B. licheniformis* subclades (Connor et al., 2010). This suggested that a classification taking into consideration the proportion of UFAs is possible, but as strains used for the study of Kaneda have been reclassified and that analytical tools of FA evolved, the Kaneda classification have to be reconsidered.

1-2-2 discrimination on Predominant FA

Based on the FAs profiles in *Bacillus* species, Kaneda *et al.* suggested a classification of *Bacillus* genus species into 6 groups (Kaneda, 1977), from A to F, displaying specific predominant FAs combined with different level of UFAs proportions (Figure 1).

In this classification, psychrotolerant strains belong to the group F having the anteiso-C_{15:0} as major FA. Studies on several psychrotrophic strains, *B. globisporus*, *B. psychrophilus* and *B. pasteurii* confirmed that the major fatty acid was anteiso-C_{15:0} (Yoon et al., 2001). Another study also showed that anteiso-C_{15:0} was the predominant FA in *B. globisporus*, *B. psychrophilus* and in another strain *B. insolitus* (Kaneda et al., 1983) . Even if all these species now belong to another genus, the *Sporosarcina* genus, these studies was used to identify new psychrotolerant species. Indeed, a novel psychrotolerant species isolated from Bering sea, *B. beringensis* displayed anteiso-C_{15:0} and iso-C_{15:0} as major FAs (Yu et al., 2011). But, the predominant FAs appeared to be iso-C_{15:0} and iso-C_{16:1} in *B. cerebensis* described as a psychrotolerant species (Reddy et al., 2008) suggesting that some exception exists.

Studies of *Bacillus cereus sensu lato* strains showed that *B. cereus sensu stricto*, *B. thuringensis*, *B. anthracis* display the FA iso-C_{15:0} as major FA (Song et al., 2000). Further studies strengthened this observation for *B. cereus sensu stricto* strains (de Sarrau et al., 2012c, Brillard et al., 2010c). Moreover, a novel thermophilic specie, *B. cytotoxicus*, belonging to *Bacillus cereus sensu lato*, was described for having iso-C_{15:0} and anteiso-C_{15:0} as major FAs (Guinebretière et al., 2013). This is in agreement with the Kaneda grouping of *Bacillus cereus sensu lato* species to group E (Figure 1).

In the thermophilic *B. stearothermophilus* (since reclassified as *Geobacillus stearothermophilus*), belonging to the Kaneda group C, the major FAs are branched-chain iso-C_{15:0} (Cho and Salton, 1966, Yao et al., 1970). A thermotolerant specie, *B. cytotoxicus* was also shown to be display as predominant FA, the branched-chain iso-C_{15:0} (Guinebretiere et al., 2013a).

In *B. subtilis* and *B. megaterium*, belonging to the Kaneda group A, studies showed that the anteiso-C_{15:0} was the major FA (Cybulski et al., 2002, Kämpfer, 1994).

This observation does not appear to be specific to the *Bacillus* genus. Indeed, some thermophilic Clostridia species from different thermal niches were shown to display different predominant FA types (Chan et al., 1971).

So, regarding the predominant FA it seems to be possible to classify the species of the genus *Bacillus*. But as explained before, the FA analytical tools evolved and some exceptions about the Kaneda classification does exist, so this classification has to be updated.

1-2-3 Taxonomy and identification of Bacillus species

All the properties described before have therefore been used for strain identification. The tools used for FA-based taxonomy have been standardized. For FAs pattern analysis, the fatty acids are extracted in the form of FA methyl esters (FAMES). Cells are harvested after growth on Trypticase soy broth agar for 24 h at 28°C, then saponification, methylation, and extraction are performed as recommended by Sherlock Microbial Identification System protocol (MIDI) as previously described (Guinebretiere et al., 2013a, Connor et al., 2010, Sasser, 1990). This MIDI method has been commonly used this last decade to discriminate species and perform taxonomy.

Song *et al.* used this method to identify the cellular fatty acid (CFA) profiles of spores and vegetative cells of some aerobic endospore-forming Bacilli (Song et al., 2000). This study

showed that FA pattern analysis of vegetative cells or spores are interesting tools for taxonomy of *Bacillus* genus strains. MIDI method was also included in the identification schema to describe new species of the genus (Yu et al., 2013, Reddy et al., 2008, Kosowski et al., 2014, Subhash et al., 2014, Logan et al., 2009) However, Kämpfer et al. demonstrated that even if there is a tendency for *Bacillus* species to conserve a typical pattern enabling to classify these species in different groups, it remains some heterogeneity of fatty acid profiles within several species (Kämpfer, 1994).

Based on all these studies, we performed an analysis of published data to determine if key elements in FA patterns could allow the discrimination of species of the *Bacillus* genus.

1-2-3-1 Bacillus cereus sensu lato classification

The taxonomy of *B. cereus sensu lato (sl)* based on predominant FA has to be separated from that of the other *Bacillus* strains. Indeed, as previously noticed *B. cereus sl* strains display a significant amount of FAs with 12 and 13 carbons (more than 10% of total FAs) (de Sarrau et al., 2012c, Brillard et al., 2010c, Guinebretiere et al., 2013a) We investigated if it was possible to discriminate strains of *B. cereus sl* (Table 2) on the basis of predominant FA. All the FAs patterns were obtained using the MIDI method, and represent published or unpublished data of INRA UMR408 laboratory.

Fortunately, *B. cereus sl* consist in 7 phylogenetic group characterized by different ranges of growth temperature (Guinebretière et al., 2008), exhibiting 5 major thermotypes : thermotolerant (group VII), mesophilic (group I, III and IV), mesophilic-psychrotolerant intermediary (group V), mesophilic-psychrotolerant intermediary to psychrotolerant (group II) and psychrotolerant (group VI) strains, as mentioned in Table 1. Moreover Tmin and Tmax of group III strains are higher than for other mesophilic strains; Tmin and Tmax of group VI strains are lower than for other psychrotolerant strains. We took into account this physiological particularity in our investigations. At least two independent replicates were done for each phylogenetic group (I to VII).

The predominant FA were identified for each phylogroup in *B. cereus sl*. This FA appeared to vary according to the phylogenetic group. It was clearly identified to be: the iso-C15:0 in phylogenetic groups VII (*B. cytotoxicus*), the iso-C15:0 together with the iso-C13:0 in phylogenetic groups II to V, the iso C15:0 together with the C16:1 and iso-C13:0 for group I (*B. pseudomyoides*) and the iso-C13:0 for group VI (*B. weihenstephanensis*, *B. mycoides*).

Interestingly the predominance of iso-C13:0 is rare in the other strains of the genus *Bacillus*. In *B. cereus* *sl*, it remained among major FAs even if it was not the predominant FA. Interestingly, from a group to another group there was an anti-correlation between the abundance of the iso-C13:0 and that of iso-C15:0. Thus, we take an interest in the i15/i13 ratio defined as the iso-C15:0 FA proportion divided by the proportion of the iso-C13:0 FA. This ratio was calculated for strains belonging to each group and was represented in figure 2-A.

Table 2: Strains of *B. cereus* *sl* used for this study

| Strains | Phylogenetic group | Thermotype | Growth range T°C | References |
|------------------------|--------------------|------------|------------------|------------------------------|
| Bp DSM 12442T | I | M | 10-43 | (Guinebretière et al., 2013) |
| Bc NVH 0861/00 | II | I | 8-40 | (Guinebretière et al., 2013) |
| Bc RIVM BC 120 | II | P | 7-40 | (Guinebretière et al., 2013) |
| Bt IEBC T61001 | II | P | 7-40 | (Guinebretière et al., 2013) |
| Bc PHLS F4810/72 | III | M+ | 15-45 | (Guinebretière et al., 2013) |
| Bc ATCC 10987 | III | M+ | 15-45 | (Guinebretière et al., 2013) |
| BcT ATCC 14579 | IV | M | 10-45 | (Guinebretière et al., 2013) |
| BtT CIP 53137 | IV | M | 10-43 | (Guinebretière et al., 2013) |
| Bc INRA 15 | V | M | 10-40 | (Guinebretière et al., 2013) |
| Bc NVH 141/1/01 | V | I | 10 -40 | (Guinebretière et al., 2013) |
| Bt IEBC T24001 | V | M | 10 -40 | (Guinebretière et al., 2013) |
| Bc UH TSP9 | V | P | 10 -40 | (Guinebretière et al., 2013) |
| Bw WSBC 10204 BwT | VI | P | 7-37 | (Guinebretière et al., 2013) |
| Bw KBAB4 | VI | P | 7-37 | (Guinebretière et al., 2013) |
| BmT DSM 2048 (inra_74) | VI | P | 7-37 | (Guinebretière et al., 2013) |
| Bcyt NVH 391-98 | VII | T | 20-50 | (Guinebretière et al., 2013) |
| B.cyt INRA AF2 | VII | T | 20-50 | (Guinebretière et al., 2013) |
| Bcyt NVH883/00 | VII | T | 20-50 | (Guinebretière et al., 2013) |
| Bcyt CVUAS2833 | VII | T | 20-50 | (Guinebretière et al., 2013) |
| Bcyt 08CEB44BAC | VII | T | 20-50 | (Guinebretière et al., 2013) |
| Bcyt 08CEB56BAC | VII | T | 20-50 | (Guinebretière et al., 2013) |

M: mesophilic; I: mesophilic-psychrotolerant intermediary; P: psychrotolerant; T : thermotolerant; Bc : *B. cereus* *ss*; Bcyt: *B. cytotoxicus*; Bm: *B. mycoides*; Bp: *B. pseudomycoides*; Bt: *B. thuringiensis*; Bw: *B. weihenstephanensis*.

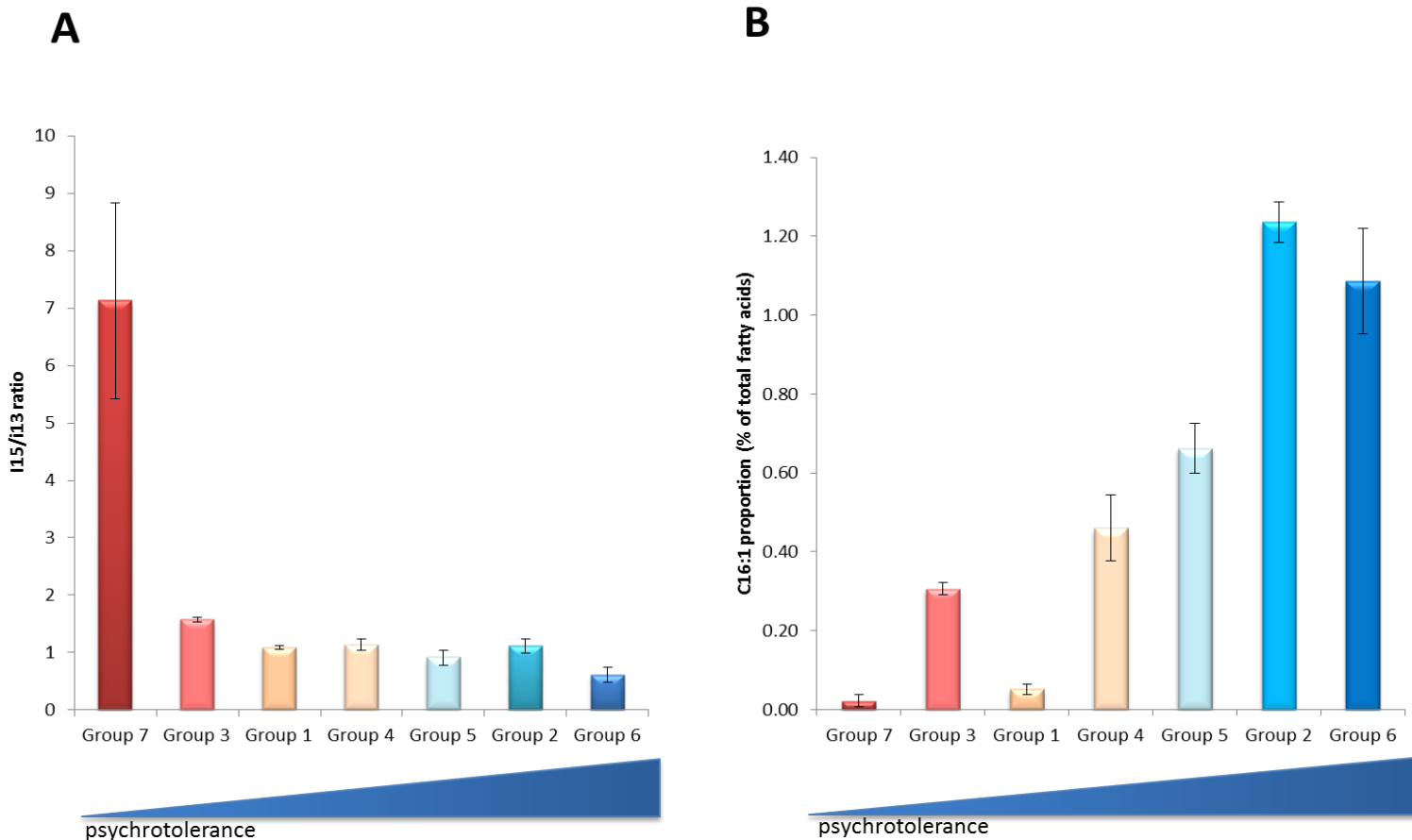


Figure 4: i15/i13 ratio (A) and proportion of C16:1 (n-11) (B) of strains belonging to the different phylogenetic groups of *B. cereus sl.*

Figure 4A shows that the i15/i13 ratio was very high in the thermotolerant strains of the group VII (7.13 ± 1.7 % of total FAs) but, this ratio tended to decrease with psychrotolerance. For instance, in the psychrotolerant strains of the group VI, the i15/i13 ratio was low being largely inferior to 1 (0.61 ± 0.13). Concerning the group II, containing mesophilic strains with a tendency to thermotolerance, the ratio was largely superior to 1 (1.57 ± 0.04 of total FAs). For the other mesophilic strains, this ratio keeps the value of 1. It was previously shown that the proportion of i13 was strongly reduced in a *B. cereus* model strain mutant compared to its parental strain during growth at low temperature (Brillard et al., 2010c). Because the i15 proportion was unchanged, the i15/i13 ratio was much higher in the mutant than in the parental strain. Interestingly, this mutant also displayed growth impairment at low temperature, emphasizing the putative role of a low i15/i13 ratio for psychrotolerance ability. All these data strongly suggest that this criteria i15/i13 ratio, could allow discriminating *B. cereus sl.* strains into psychrotolerant, mesophilic and thermotolerant strains.

Another FA, C16:1 (n-11) also appeared to be an indicator for classification of phylogenetic groups in *B. cereus* ss. Figure 4B shows the proportion of this FA for strains of the different phylogenetic groups of *B. cereus* sl. Psychrotolerant strains of the group II and VI displayed the highest proportion of this UFA, with respectively 1.14 ± 0.05 and 1.09 ± 1.13 of total FAs while thermotolerant strains of the group VII displayed a very low proportion of this UFA (0.02 ± 0.01). Moreover, the C16:1 (n-11) proportion tended to decrease with thermotolerance (proportion in group II > proportion in group V > proportion in group IV > proportion in group 3). In the group III, this ratio was higher (0.31 ± 0.02) than in group I (0.05 ± 0.01), but still lower than in other mesophilic strain.

This FA also seems to be a good indicator for predictive growth properties of strains of *B. cereus* sl.

Thus, these data suggest that on the basis of FA predominance and of key FAs ratios, it is possible to predict the thermotype of strains of *Bacillus cereus* sl. Even if this study strengthens some observations assumed by Kaneda, it highlights the key role of the iso-C_{13:0}, a specific FA of *Bacillus cereus* sl strains, in classification of the strains of this group. Further investigations might be done on a largest panel of *B. cereus* sl strains in order to validate the conclusions made here. Another property interesting to check would be the effective changes in FA predominance for *B. cereus* sl strains when incubated at their minimal (T_{min}) or maximal growth temperature (T_{max}), compared to the result presented here.

1-2-3-2 Bacillus genus species classification excluding B. cereus sl

We investigated on how to discriminate the other species or groups of the genus *Bacillus*. Two methods were used for this study: the heat adaptation index and the a15/i15 ratio.

Connor *et al.* revealed that some FAs allow discriminating *B. subtilis* and *B. licheniformis* strains belonging to different ecotypes (Connor *et al.*, 2010) by calculating the heat adaptation index. To determine this parameter, two factors have to be calculated : (i) the sum of the proportion of some FAs allows to obtain the high temperature adaptation factor (n14:0; n16:0 ; i14:0; i15:0; i16:0 and i17:0), (ii) the sum of the proportion of a15:0; a17:0; n16:1; i17:1 (n-10) and 16:1 ω7c alcohol, allow determining the low-temperature adaptation factor. The heat adaptation index (HAI) is obtained by dividing the high temperature adaptation factor by the low-temperature adaptation factor (Connor *et al.*, 2010).

$$\text{HAI} = \frac{p(\text{n14:0}) + p(\text{n16:0}) + p(\text{i14:0}) + p(\text{i15:0}) + p(\text{i16:0}) + p(\text{i17:0})}{p(\text{a15:0}) + p(\text{a17:0}) + p(\text{n16:1}) + p(\text{i17:1}) + p(\text{16:1 } \omega\text{7c alcohol})}$$

p is the proportion of the FA

We therefore tried to verify if it was possible to discriminate the species of the *Bacillus* genus (except *B. cereus* *sl*) using this method. For this, FAs patterns data realized using the MIDI method data, and obtained from the Song *et al.* study (Song *et al.*, 2000) were used (see Table 3), including species later characterized to belong to different other genus e.g. *Sporosarcina*, *Paenibacillus*, *Geobacillus*, *Lysinibacillus* (Yoon *et al.*, 2001, Pettersson *et al.*, 1999, Nazina *et al.*, 2001, Ahmed *et al.*, 2007).

This method was compared to another analysis based on predominant FAs (FAs with the highest proportion) and previously used for *Bacillus* species classification (Kämpfer, 1994). For the comparison of these methods, we used the FAs patterns data realized by song *et al* (Song *et al.*, 2000). For all the *Bacillus* species we studied, the predominant FAs were iso-C15:0 or anteiso-C15:0 as previously observed (Kämpfer, 1994). It was also previously demonstrated that the a15:0 FA was the major FA in the genus *Bacillus* psychrotolerant strains (Kaneda, 1977). When observing the FA pattern of song *et al.* tested strains (Song *et al.*, 2000), it appeared that there was an anti-correlation between the i15:0 proportion and the a15:0 proportion. So, in the same logic as described previously for *B. cereus* *sl* members, the a15:0/ i15:0 ratio was calculated for all the genus *Bacillus* strains studied by song *et al*, 2000. The data obtained was compared to those calculated with the heat adaptation index (Figure 3-A and 3-B).

Even if the methods of FAs analysis used were different between Kämpfer and Song data, when calculating the a15:0/ i15:0 ratio, the distribution of the strains was the same than that observed by Kämpfer.

In both HAI or a15:0/ i15:0 representations, it was possible to discriminate 3 categories of strains: thermophilic, mesophilic, and mesophilic strains. The psychrotolerant strain *Sporosarcina globispora* (ancient name *B. globisporus* (Yoon *et al.*, 2001)) displayed the lowest HAI (Figure 3-A) and highest a15:0/ i15:0 ratio (figure 3-B). Psychrotolerant strains of the genus *Paenibacillus* appeared also to display the same pattern in both representations, low HAI (<1) and high a15:0/ i15:0 ratio (>3).

Table 3: Strains used for *Bacillus* genus classification using FA composition, respective reviewed name and growth temperature range obtained from the Bergey's manual (Paul Vos, 2011) and the database ABIS encyclopedia.

| Strains | Reference | Growth temperature range |
|---|---------------------|--------------------------|
| <i>Bacillus subtilis</i> 170311 | (Song et al., 2000) | ND |
| <i>B. aminovorans</i> 94021 T | (Song et al., 2000) | 5-10 to 37 °C |
| <i>B. amyloliquefaciens</i> 170517 | (Song et al., 2000) | 15 to 50 °C |
| <i>B. amyloliquefaciens</i> 170518 | (Song et al., 2000) | 16 to 50 °C |
| <i>B. amyloliquefaciens</i> 94022 | (Song et al., 2000) | 17 to 50 °C |
| <i>B. atropheus</i> 95007 T | (Song et al., 2000) | 10 to 55 °C |
| <i>B. atropheus</i> ATCC 9372 | (Song et al., 2000) | 10 to 55 °C |
| <i>B. badius</i> 94024 | (Song et al., 2000) | 15 to 50 °C |
| <i>B. circulans</i> CCTCCAB 94026 | (Song et al., 2000) | 5-20 to 35-50 °C |
| <i>B. coagulans</i> CCTCCAB 92066 | (Song et al., 2000) | 15-25 to 55-60°C |
| <i>B. firmus</i> CCTCCAB 170519 | (Song et al., 2000) | 5-20 to 40-50 °C |
| <i>B. firmus</i> CCTCCAB 94028 T | (Song et al., 2000) | 5-20 to 40-50 °C |
| <i>B. globisporus</i> CCTCCAB 94031 T (<i>Sporosarcina globispora</i>) | (Song et al., 2000) | 0-3 to 25-30 °C |
| <i>B. insolitus</i> CCTCCAB 94032 T (<i>Psychrobacillus insolitus</i>) | (Song et al., 2000) | 0-5 to 25 °C |
| <i>B. lentimorbus</i> CCTCCAB 94034 T (<i>Paenibacillus lentimorbus</i>) | (Song et al., 2000) | 20 to 35 °C |
| <i>B. lentus</i> CCTCCAB 94035 T | (Song et al., 2000) | 10 to 40 °C |
| <i>B. licheniformis</i> CCTCCAB 170513 | (Song et al., 2000) | 15 to 50-55°C |
| <i>B. licheniformis</i> CCTCCAB 170514 | (Song et al., 2000) | 16 to 50-55°C |
| <i>B. licheniformis</i> CCTCCAB 94036 T | (Song et al., 2000) | 17 to 50-55°C |
| <i>B. megaterium</i> 170201 | (Song et al., 2000) | 3-20 to 35-45 °C |
| <i>B. megaterium</i> 170505 | (Song et al., 2000) | 3-20 to 35-45 °C |
| <i>B. megaterium</i> CCTCC AB 92075 T | (Song et al., 2000) | 3-20 to 35-45 °C |
| <i>B. mojavensis</i> CCTCC AB 96001 T | (Song et al., 2000) | 10 to 55 °C |
| <i>B. niacini</i> CCTCC AB 95011 T | (Song et al., 2000) | 10 to 40 °C |
| <i>B. pumilus</i> CCTCC AB 94044 T | (Song et al., 2000) | 5-15 to 40-50°C |
| <i>B. simplex</i> CCTCC AB 94045 T | (Song et al., 2000) | 20 to 40 °C |

| Strains | Reference | Growth temperature range |
|---|---------------------|--------------------------|
| <i>B. sphaericus</i> 170511 (<i>Lysinibacillus sphaericus</i>) | (Song et al., 2000) | 10-15 to 30 45°C |
| <i>B. sphaericus</i> 170512 (<i>Lysinibacillus sphaericus</i>) | (Song et al., 2000) | 10-15 to 30 45°C |
| <i>B. sphaericus</i> CCTCC AB 92073 T (<i>Lysinibacillus sphaericus</i>) | (Song et al., 2000) | 10-15 to 30 45°C |
| <i>B. stearothermophilus</i> CCTCC AB 92070 T (<i>Geobacillus stearothermophilus</i>) | (Song et al., 2000) | 30-45 to 65-75 °C |
| <i>B. subtilis</i> CCTCCAB 92068 T | (Song et al., 2000) | 5-15 to 40-45 °C |
| <i>B. subtilis</i> 170221 | (Song et al., 2000) | 5-15 to 40-45 °C |
| <i>B. subtilis</i> 170312 | (Song et al., 2000) | 5-15 to 40-45 °C |
| <i>B. thiaminolyticus</i> CCTCC AB 95017 T (<i>Paenibacillus thiaminolyticus</i>) | (Song et al., 2000) | 20 to 45 °C |
| <i>Brevibacillus agri</i> CCTCC AB 95005 T | (Song et al., 2000) | 5-20 to 40 °C |
| <i>Brevibacillus brevis</i> CCTCC AB 94025 T | (Song et al., 2000) | 10-35 to 40-50 °C |
| <i>Paenibacillus amylolyticus</i> CCTCC AB 95019 T | (Song et al., 2000) | 10-15 to 40 °C |
| <i>Paenibacillus larvae</i> CCTCC AB 94033 T | (Song et al., 2000) | 20 to 40 °C |
| <i>Paenibacillus pabuli</i> CCTCC AB 95012 T | (Song et al., 2000) | 5-10 to 35-40 °C |
| <i>Paenibacillus peoriae</i> CCTCC AB 95013 T | (Song et al., 2000) | 5-10 to 35-45 °C |
| <i>Paenibacillus polymyxa</i> 170507 | (Song et al., 2000) | 5-10 to 35-45 °C |
| <i>Paenibacillus polymyxa</i> 170508 | (Song et al., 2000) | 5-10 to 35-45 °C |
| <i>Paenibacillus polymyxa</i> CCTCC AB 92076 T | (Song et al., 2000) | 5-10 to 35-45 °C |

Paenibacillus genus is a genus very close to the *Bacillus* genus, comprising species previously described as members of the *Bacillus* genus (Shida et al., 1997, Pettersson et al., 1999, Uetanabaro et al., 2003, Heyndrickx et al., 1995). Indeed, *Paenibacilli* are aerobic, Gram-positive bacteria related to *bacilli* but differing from *Bacillus* strains in the DNA encoding their 16S rRNAs (Paul Vos, 2011). This genus mostly contains psychrotolerant strains (Ivy et al., 2012).

B. simplex has been extensively described for the high ability of its strains to adapt to different environmental conditions (Sikorski and Nevo, 2007, Sikorski and Nevo, 2005, Sikorski et al., 2008). The strain studied here displayed a high a15:0/ i15:0 ratio and low and HAI. As this strain is the type strain of *B. simplex* and because Bergey's manuel described this strain as able to grow at 20 and 30°C but not at 45°C (Paul Vos, 2011), we can suggest that the strain presented here is a psychrotolerant *B simplex* strain.

Several strains that displayed a HAI comprised between 1 and 2, and a15:0/ i15:0 around the value of 1 were considered as mesophilic strains. In the Figure 5, the location of mesophilic species like *B. lentus*, *B firmus*, *B. amyloliquefaciens*, *B. niacini*, *P. lentimorbus*, *B mojavensis*, *Br. brevis* and *Br. agri*, of some strains of *B. megaterium* and *B. subtilis* (Paul Vos, 2011), is consistent with this observation.

For both representations, *Geobacillus sterarothermophilus* (old name: *B. sterarothermophilus* (Nazina et al., 2001)) displayed a high HAI (Figure 5A) and a low a15:0/ i15:0 ratio (Figure5B). This is consistent because this strain is thermophilic. *B. licheniformis* and some *B. megaterium* are known to be thermophilic strains. Bergey's manuel described these strains as phylogenetically closely related to *G. stearothermophilus*. In our study, their distribution in both representations was close to *G. stearothermophilus*, except for some *B. megaterium* strains.

As tools for taxonomy evolve, numerous strains previously classified into the *Bacillus* genus and later moved in other genus displayed an abnormal distribution among the studied strains (Figure 5). For example, *B. sphearicus* is described as a mesophilic strain (Paul Vos, 2011) but its high heat adaptation index and low a15/i15 indicates a profile similar to the thermophilic strains. A recent study proposed its reclassification of this species in the *Lysinibacillus* genus (Ahmed et al., 2007).

Strains of *B. circulans* were described as psychrotolerant strains and some were moved into the *Paenibacillus* genus (Ivy et al., 2012, Paul Vos, 2011), it remains however some thermotolerant strains (Hazem and Manar, 2003). The strain used in this study did not display psychrotolerant character. So, it is possible to hypothesize that the strain studied is a thermotolerant one.

B. insolitus strains is a psychrophilic strain (Paul Vos, 2011), but as *Lysinibacillus sphearicus*, have a profile similar to the thermophilic strains, for this strain also a reclassification was also recently proposed in the genus *Psychrobacillus* genus.

The inconsistencies described above may be indicative as they may help in excluding some strains from a specific group and therefore efficiently define a group of strains.

However *B. coagulans*, moderately thermophilic species here displayed a very low HAI and a a15:0/ i15:0 ratio quite high. This observation was made before during Kämpfer studies. When analyzing the FA profile, it appeared that this strain studied displayed two major FA: an a17:0 and an i17:0. This is different compared to the other strains of the group and may explain its location among psychrotolerant strains.

Other exceptions do exist: a novel thermotolerant strain, *B. thermotolerans* display an iso-C16:0 as major FA instead of a typical iso-C15:0 (Yang et al., 2013).

In conclusion, the two methods tested here can be used for the classification of *Bacillus* species in thermotypes. However, the fact that the a15:0 and i15:0 are also in a ratio in the HAI and that the two methods here gave the same global classification may indicate that the other FAs utilized in the HAI have a minor role for the thermotype determination. Moreover, the classification based on the a15:0/ i15:0 ratio seems to be a little more discriminating because the species belonging to the same genus (i.e. *Peainibacillus*) appeared closer to each other in this representation compared to the HAI representation.

Whatever the method used, it is important that the taxonomic group studied display at least the same dominant FAs in order to avoid bias of analysis (i.e. *B. cereus* s.l). Indeed, even if FA predominance seems to be an interesting tool for classification of strain, some heterogeneity remains between FA profiles within *Bacillus* species which may lead to an unacceptable classification of strains with different thermotype belonging to this genus. That is why it is necessary to complete FA pattern analysis with a genomic analysis data to establish a better identification of *Bacillus* strains.

1-3 FA composition changes for *Bacillus* adaptation

1-3-1 Changes observed during food processing

Several members of the *Bacillus* genus are responsible of foodborne illness outbreak (FBI) notably *B. cereus sensu stricto* strains (Stenfors Arnesen et al., 2008b), *B. cytotoxicus* (Guinebretiere et al., 2013a) and some psychrotolerant strains (Stenfors and Granum, 2001).

Here we discuss about the faculty of *Bacillus* species to change their FA during the food processing.

A study revealed that *B. cereus* strains isolated from whey manufacturing process were different from the isolates originating from the raw materials of cheese making, considering their whole FA profiles (Pirttijärvi et al., 1998). A similar observation was done in another industrial process such as food grade paper and paperboard production (Pirttijarvi et al., 2000). Indeed, it was shown that the FA composition of industrial isolates deviated so much from that of the commercial database that the strains were not or poorly recognized as *B. cereus*. All these data clearly suggest that *B. cereus* strains are able to modulate their FA composition depending on the food processing.

1-3-2 Changes depending on the growth medium composition

The growth medium (including food) may also influence the FA composition of several strains of the *Bacillus* genus. for instance, UFAs from spinach or from the growth medium changed the FA membrane composition of *B. cereus* (de Sarrau et al., 2013d) leading to an improvement of growth at low temperature and anaerobiosis.

Strains of *B. cereus* isolated from rice were also tested for their growth ability at reduced temperature in rice starch characterized by a low water activity (Haque and Russell, 2004b). These conditions stimulated the growth of the tested strains at low Aw and temperature when compared with strains grown without rice starch by modifying the FA composition.

When the growth is performed in presence of molecules such as butyrate, isobutyrate, valerate, and isovalerate, compounds that are specific precursors for branched-chain FA biosynthesis, it was shown that there is an increase in branched-chain FA of *B. thuringensis* spores (Nickerson and Bulla, 1980)..

1-3-3 Changes depending on the temperature

Effect of temperature on FA composition of *Bacillus* has been extensively studied for years (Fulco, 1967). When confronted to a change of temperature, species of the *Bacillus* genus modify their FA composition (de Sarrau et al., 2012c, Aguilar et al., 2001b). These changes often involved branched-chain and unsaturated FAs. When temperature was raised to 60, 70 or 80 °C, an increase in the iso-branched-chain FA iso-C17 and the linear C16:0 FA was

observed in thermophilic species such as *B. caldolyticus* and *B. caldotenax* (Weerkamp and Heinen, 1972).

Bacillus genus species isolated under anoxic conditions from an intertidal mudflat of the northwest German coast were analyzed for temperature-induced changes in their whole cell FA patterns (Freese et al., 2008). This study demonstrated that decreasing growth temperature induced an increase in monounsaturated FAs and a decrease in saturated straight chain FAs. In addition to these changes, branched-chain FAs (whether iso or anteiso-branched-chain FAs) changes proportion increased with decreasing temperature (Freese et al., 2008).

Similarly, an increase proportion of anteiso-branched and unsaturated FAs was observed *B. simplex* strains isolated from specific habitats of a Canyon in Israel when growth was done at low temperature (20°C) (Sikorski et al., 2008). A significant decrease of the relative concentration of all unbranched or iso-branched FAs was also observed. However, at high temperature (40°C), the iso-branched FAs proportion increased, whereas the anteiso-branched and unsaturated FAs proportions decreased (Sikorski et al., 2008).

Investigations about *B. subtilis* and *B. cereus* adaptation to low temperature revealed the key role that UFAs play for (Aguilar et al., 2001b) for membrane fluidity homeostasis at reduced temperature (Chazarreta Cifre et al., 2013, Haque and Russell, 2004b, Brillard et al., 2010c). In both species, studies revealed that only anteiso/iso ratio increased in a higher proportion in *B. subtilis* when low temperature was combined with anaerobiosis (de Sarrau et al., 2012c, de Sarrau et al., 2013a, Beranova et al., 2010), indicating a lack of desaturation in this growth condition.

In conclusion, the main changes observed in fatty acid composition of the genus *Bacillus* for low and high temperature are represented in Figure 6. For low temperature adaptation, anteiso- and unsaturated FAs proportion are increased while iso- and saturated FAs proportion are decreased. In contrast, SFAs proportion increases, anteiso and unsaturated FA proportions decrease at high temperature.

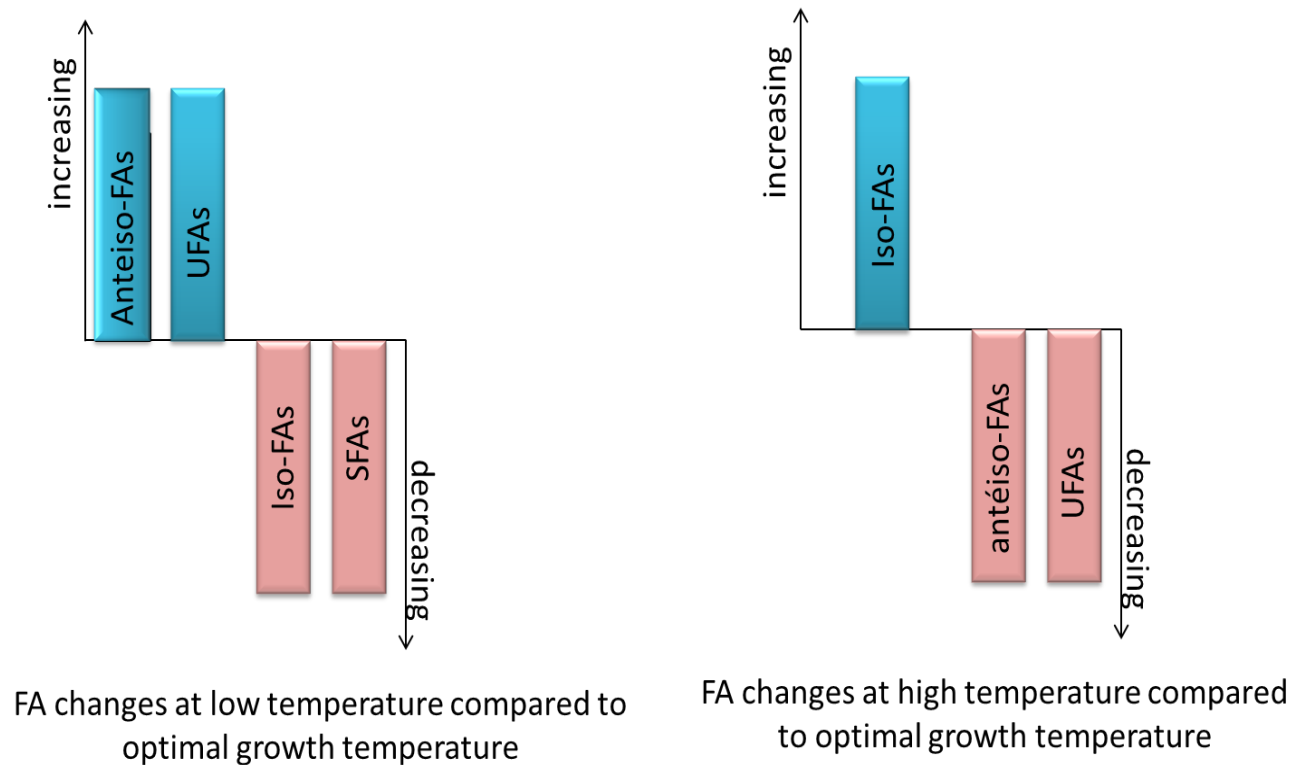


Figure 6: changes in FA composition depending on temperature

1-3-4 changes induced by other conditions

Several other abiotic factors are known to induce modification in the FA composition of *Bacillus* species.

When comparing FA profile of *B. alkaliphilic* grown at pH 10, the ratio of UFAs and anteiso/iso branched-chain FA was lower than when grown at pH 7 (Yumoto et al., 2000). Moreover, a transcriptomic analysis during a sorbic acid stress in *B. subtilis* revealed that genes of FA biosynthesis were upregulated indicating a plasma membrane remodeling in the stressed cells (Ter Beek et al., 2008).

Gamma irradiation treatment also induces changes in FA composition of *Bacillus* species. In *B. cereus*, this treatment increase the proportion of UFAs (Ayari et al., 2009).

Pressure is another influencing factor: *B. subtilis* cells grown at 1013 versus 50 mbar revealed a decrease in the ratio of unsaturated to saturated FAs but an increase in the ratio of anteiso- to iso-FAs (Fajardo-Cavazos et al., 2012).

Some nanoparticles such as carbon nanotubes may be toxic for bacteria. When *B. subtilis* was exposed to carbon nanotubes, an increased proportion of branched-chain fatty acids and a

decreased level of straight-chain fatty acids were observed. These changes in FAs composition were favorable to counteract the toxic effect of carbon nanotubes.

So, when facing various stressful environmental conditions, such as those described above, *Bacillus* species adapt their FA composition, by increasing the proportion of FA with lower melting points.

1-3-5 Production of FA with antimicrobial activity

In presence of other organisms, *Bacillus* species are able to synthesize some special FAs which are components of molecules displaying biocide abilities. For example, surfactin is a cyclic lipopeptide containing a β -hydroxy FA, but no di-sulfide bridges or sugar residues. This molecule, produced by *B. subtilis subsp. subtilis* and *B. licheniformis* has antimicrobial properties (Compaore et al., 2013).

B. amyloliquefaciens, is able to synthesize 26 types of surfactin acting as potential antifungal metabolites. Among these 26 surfactin types, several new cyclic as well as acyclic surfactin variants were identified, based on the variation in the β -hydroxy fatty acid (β -OH FA) chain length and/or in amino acid positions 4, 5, 6, and 7 (Pathak et al., 2014). Other surfactins such as ones with long fatty acid chain (C14 and C15), characterized in *B. subtilis Cohn* (S499 strain), displayed insecticide effect on the fruit fly, *Drosophila melanogaster* (Assie et al., 2002).

1-3-6 Changes depending on the bacterial cell state: spores and vegetative cells

Sporulation and germination of *Bacillus* species also induce changes in FA pattern. Indeed, it was shown that a de novo FA synthesis was required for establishment of cell type-specific gene transcription during sporulation in *B. subtilis*, (Schujman et al., 1998).

B. weihenstephanensis KBAB4 spores obtained at 30 °C displayed almost the same profile than vegetative cells, containing the i13:0 and the i15:0 as major FA, similarly as the other members of *B. cereus* *sl* (Planchon et al., 2011). But after sporulation at 10 °C, the spores displayed another pattern predominated with the i15:0, the a15:0, i17:0 and i13:0 (Planchon et al., 2011).

In a study of about 50 different species of *Bacillus* genus species, it was shown that spores FAs content and cellular FAs contents were very similar (Song et al., 2000), but the

proportion of branched-chain FA (and more particularly the i15:0, the a15:0 and i17:0) was higher in spores than in the vegetative cells. Moreover, some FA present in vegetative cells was only at trace level in spores e.g. 3OH-C14:0 acid, iso-C16:1 ω 7c and anteiso-C17:1 ω 5c. In *B. megaterium* sporulation induced synthesis of i14:0 branched-chain FA in addition to the major FA i15:0 and consequently, an increase of branched-chain FA proportion was observed in spores (Scandella and Kornberg, 1969). When the spores germinated, the FA proportion turned back to that of the vegetative cells (Scandella and Kornberg, 1969).

Thus, the conclusion of this chapter is that *Bacillus* genus species are able to display different FA pattern depending on environmental factor mainly related to adaptation and survival (Figure 7). This ability of *Bacillus* species to easily change their FAs profiles may explain why *Bacillus* species are known to be ubiquitous. Even if the FA composition of a *Bacillus* species changes in response to environmental fluctuations, some features of the FA pattern may be conserved, correlated to the ability to develop in a given environment. Therefore, it is important to distinguish a short term change in FA composition (short-time adaptation) and a long term change in FA composition (long term adaptation).

Some precautions should thus be taken, because these changes may lead to biases during identification of strains. It is therefore important to combine genetic comparison with FA pattern and define optimal growth parameters of the newly described strain before the genus and/or species affiliation.

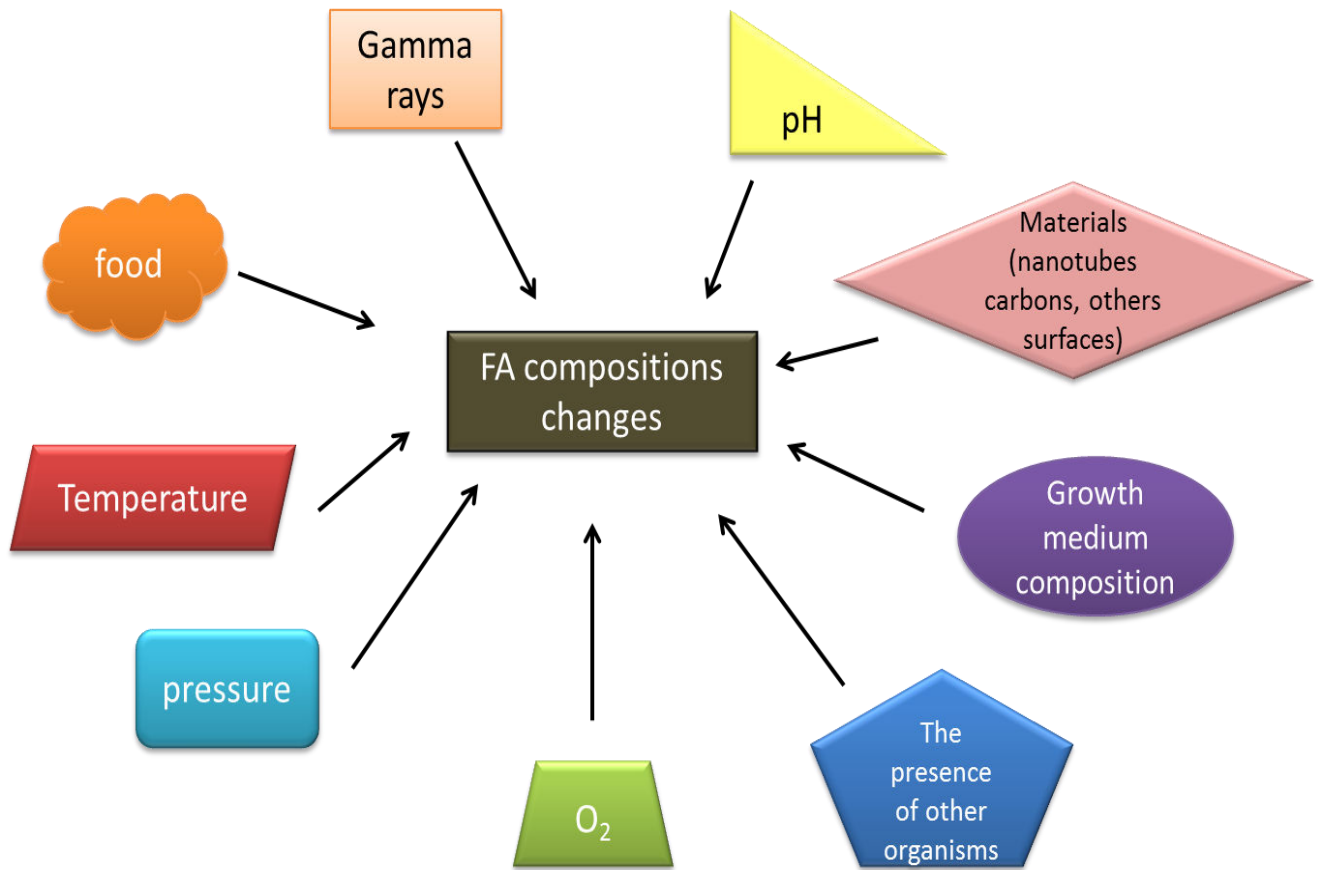


Figure 7: Environmental factors influencing the FA composition of Bacillus genus strains

2- Metabolism

2-1 FA biosynthesis in *Bacillus*

2-1-1 Biosynthesis of saturated FA

In living organisms, the fatty acid synthesis (FAS) comprises a repeated cycle of reactions consisting of the condensation, reduction, dehydration, and subsequent reduction of carbon-carbon bonds. In higher eukaryotes, these reactions are carried out by a large multifunctional protein forming the so-called type I FAS pathway (Chirala et al., 1997). FAS type II pathway reactions are catalyzed by discrete enzymes in bacteria, plant chloroplasts, and also in the eukaryotic protozoan *Plasmodium falciparum* (van Schaijk et al., 2014, Freiberg et al., 2004, Marrakchi et al., 2002). Two (or more) enzymes are available to catalyze a given step of the pathway, although these enzymes may differ in substrate specificity and hence physiological function (Rock and Cronan, 1996). *Bacillus* strains possess a highly related, and clearly identified, set of genes that carry out the reactions in the pathway.

The biosynthesis of FAs is the first step in the formation of membrane lipids, and represents a vital aspect of bacterial physiology. This pathway was extensively studied in the Gram negative *E. coli* which serves as a model for understanding Type II systems in other bacteria. The FAS cycle basic steps are common to all bacteria, and the genes encoding the enzymes are generally conserved (Marrakchi et al., 2002). We will describe the pathway that has been studied in *B. subtilis* (White et al., 2005a, Marrakchi et al., 2002)(see Figure 8).

The first group of enzymes constitutes the initiation module (Figure 6). This group is a multisubunit enzyme consisting on acetyl coenzyme A (acetyl-CoA) carboxylase (ACC), catalyzing a key step in intermediary metabolism that converts acetyl-CoA to malonyl-CoA. The overall ACC reaction requires four gene products, AccA, AccB, AccC, and AccD. The only known destination for malonyl-CoA in *E. coli* is FAS (White et al., 2005b). Malonyl-CoA is transferred to the acyl-carrier-protein synthase enzyme (ACP) by the malonyl-CoA:ACP transacylase (FabD) to form malonyl-ACP (White et al., 2005b, Marrakchi et al., 2002). Fatty acid synthesis is then initiated by the condensation of malonyl-ACP as the primer with acetyl-CoA as the acceptor.

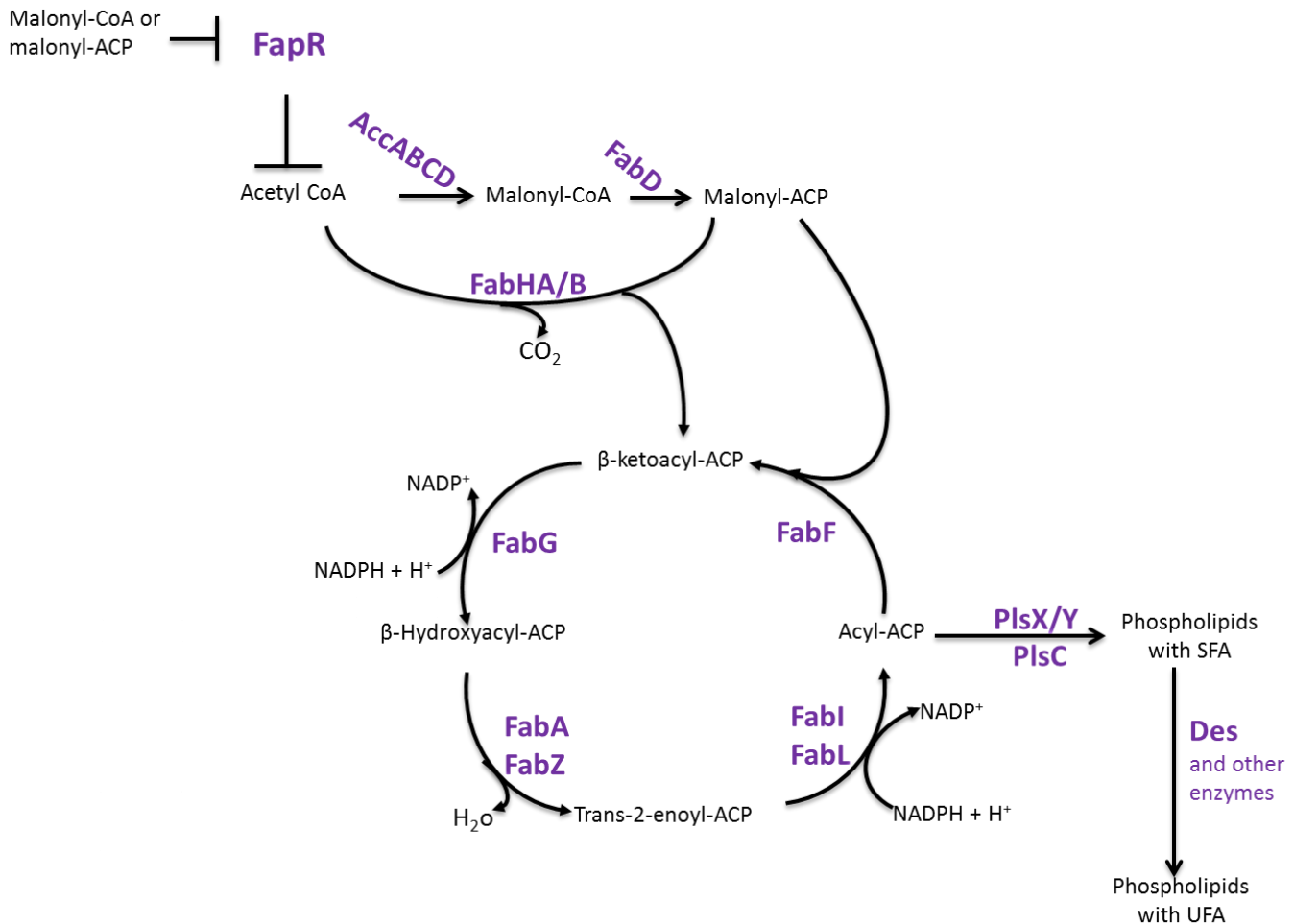


Figure 8: *Bacillus* FAS II pathway, FA integration into phospholipids and main regulators of this pathway. Enzyme are represented in bold and in purple. —|— indicates a repression

This first condensation step is catalyzed by β-ketoacyl-ACP synthase III (FabH) to form β-ketobutyryl-ACP and CO₂. This initiates a cycle of elongation of the acyl-ACP by two carbon units. A repetitive series of reactions takes place, adding two-carbon units per cycle, until a final saturated fatty acid is formed. The elongation module consists of proteins condensing an acyl-ACP primer with malonyl-ACP. The first reaction in this cycle is the NADPH-dependent reduction of β-ketoacyl-ACP to β-hydroxyacyl-ACP by β-ketoacyl-ACP reductase (FabG). The β-hydroxyl intermediate obtained is dehydrated to yield trans-2-enoyl-ACP catalyzed by β-hydroxyacyl-ACP dehydratase (FabZ). The last step of the cycle is the NAD(P)H-dependent reduction of the double bond in the trans-2-enoyl-ACP intermediate by an enoyl-ACP reductase (enoyl-ACP reductase I (FabI), enoyl-ACP reductase III (FabL)) to form an acyl-ACP. β-ketoacyl-ACP synthase II (FabF) initiates the subsequent elongation cycles by condensation of malonyl-ACP with the acyl-ACP. Indeed, in contrast to *E. coli*

which expresses both FabF and FabB for condensation steps, the sole condensing enzyme for subsequent elongation steps of FAS is FabF in *B. subtilis* (Schujman et al., 2001).

2-1-2 Branched-chain fatty acid

B. subtilis and all the *Bacillus* genus species produce mainly branched-chain FA. For the synthesis of this FA group, the same set of enzyme of FAS is used. It was shown that in *B. subtilis* two FabH isozymes exist, FabHA and FabHB which are selective for branched-chain acyl-CoA (Figure 8). Indeed, this enzyme carrying out the initial condensation reaction of fatty acid biosynthesis with acetyl-CoA as a primer, also utilizes iso- and anteiso-branched-chain acyl-CoA primers as substrates, leading to the production of primarily iso- and anteiso-branched chain fatty acids (Choi et al., 2000b). Acetyl-CoA is used as substrate only for the synthesis of straight-chain fatty acids. Branched-chain fatty acids were shown to arise from branched-chain amino acid metabolism (Kaneda, 1991, Willecke and Pardee, 1971). These branched-chain amino acids are valine, isoleucine and leucine. Their metabolism produces short-chain branched acyl-CoAs such as isobutyric-, isovaleric-, and 2-methylbutyric-CoA that are used as primers for type II FA synthesis in place of acetyl-CoA (Willecke and Pardee, 1971, He and Reynolds, 2002, Choi et al., 2000b). Isoleucine is the precursor of anteiso-branched chain FAs while leucine and valine give rise to the primers for iso-branched fatty acids (Kaneda, 1991, Kaneda, 1977). The substrate specificity of the FabH condensing enzyme is the determining factor in the biosynthesis of branched-chain fatty acids by FAS II (Choi et al., 2000b).

The branched-chain α -ketoacid decarboxylase, shown to be essential for the branched-chain FA biosynthesis, catalyzes the decarboxylation of α -ketoacids, derived from branched-chain amino acids, to generate branched-chain acyl-CoA primers (Lu et al., 2004, Willecke and Pardee, 1971). In *B. subtilis*, a mutant of the gene encoding for this enzyme was auxotroph for branched-chain FA precursors related to isoleucine, valine, leucine (Boudreaux et al., 1981, Willecke and Pardee, 1971).

2-1-3 Antibiotics targeting the FASII

Several antibiotics were identified to target key elements of the FA metabolism of *Bacillus* strains. In *B. subtilis*, the fusaricidin treatment displays an antibacterial activity by increasing

the catabolism of fatty and amino acids and strongly repressed glucose decomposition and gluconeogenesis (Yu et al., 2012).

The cerulenin is an antibiotic characterized as targeting the condensing enzyme FabF (Schujman et al., 2001). The effect of this antibiotic in *B. subtilis* is the increased incorporation of FA of shorter chain length into bacteria membrane (Porrini et al., 2014).

The elongation condensing enzymes of the FASII, FabH and FabF are the target of numerous antibiotics like thiolactomycin, Phomallenic acid C, thiotetromycin, and Tü3010 (Young et al., 2006).

The triclosan was also described to also target FASII and more precisely the enoyl-acyl carrier protein reductase FabI and FabL in *B. anthracis* and *B. subtilis* (Tipparaju et al., 2008, Porrini et al., 2014).

2-1-4 Unsaturated Fatty acid biosynthesis

As previously mentioned, a decrease of temperature results in an increase of UFAs proportion in the bacterial cells. The main objective is to maintain the membrane fluidity by using these FAs that display a lower melting point than saturated FAs.

FAS II was shown to be active during both aerobically and anaerobically pathway for UFAs synthesis in *E. coli*, as it does not require molecular oxygen (Cronan and Vagelos, 1972). But in *Bacillus* species, the two genes *fabA* and *fabB* described in *E. coli* as responsible for UFAs biosynthesis do not exist. However, the aerobic desaturation pathway for UFA biosynthesis was well described in *Bacillus* species.

B. subtilis was shown to have a single acyl-lipid oxygen-dependent desaturase, named Des (Aguilar et al., 1998), that inserts a *cis*-double bond at the $\Delta 5$ position of the acyl chains of membrane phospholipids (Kaneda, 1972). A $\Delta 5$ -desaturase was also described in *B. megaterium* (Fulco, 1967) and in *B. cereus* (Chazarreta Cifre et al., 2013). Ferredoxin and two flavodoxins (YkuN and YkuP) were identified to be redox partner of $\Delta 5$ -desaturase as they act as electron donor for the desaturation reaction (Chazarreta-Cifre et al., 2011). This suggests that these 3 proteins may function physiologically in the biosynthesis of unsaturated fatty acids in *Bacillus* species (Chazarreta-Cifre et al., 2011). The $\Delta 5$ -desaturases play a key role in low temperature adaptation (Mansilla et al., 2003, Lombardi and Fulco, 1980, Kaneda, 1972). But some species such as *B. cereus* possess another $\Delta 10$ desaturase, that inserts a *cis*-double bond at the $\Delta 10$ position of the acyl chains of membrane phospholipids, active regardless of growth temperature (Chazarreta Cifre et al., 2013).

As some species of *Bacillus* genus are facultative anaerobes (Tang et al., 2014, Yu et al., 2013, Rosenfeld et al., 2005), in anaerobic conditions *B. subtilis* and *B. cereus*, and presumably other *Bacillus* species devoided of FabA and FabB, are not able to synthesize UFAs anymore. In cold conditions this may prevent the adaptation of the membrane fluidity as in the case of *B. cereus* (de Sarrau, Clavel et al. 2012). Although in the case of *B. subtilis*, increasing the anteiso/iso ratio permits to maintain the membrane fluidity (Beranova et al., 2010)

2-1-5 Phospholipid synthesis

FAs mostly occur in the form of phospholipid and phosphatidic acid is the basic structure of the glycerophospholipids. Phosphatidic acid (PtdOH) is the biosynthetic product of the esterification of two fatty acids onto the two hydroxyl groups of glycerol-3-phosphate (G3P). G3P is a phosphate ester of the 3-carbon sugar glyceraldehyde and the only *de novo* pathway known for the synthesis of this molecule in bacteria is the reduction of dihydroxyacetone phosphate by the G3P synthase (GpsA) (Cronan and Bell, 1974, Beijer et al., 1993, Kito and Pizer, 1969, Ray and Cronan, 1987, Morbidoni et al., 1995). PtdOH biosynthesis starts with the acylation of glycerol-3-phosphate (G3P) to form 1-acyl-G3P. As for most of the Gram positive bacteria, there are two enzyme systems that carry out the first reaction in *B. subtilis*. PlsX (acyl-acyl-ACP-phosphate acyltransferase) and PlsY (acyl-phosphate-glycerol-phosphate acyltransferase) (Figure 6) (Yoshimura et al., 2007, Lu et al., 2007). The soluble enzyme PlsX converts acyl-ACP to acyl-phosphate and the membrane-associated PlsY transfers the acyl group from acyl-phosphate to glycerol 3-phosphate (Lu et al., 2007). The acylation of the 2-position of the 1-acyl-G3P is catalyzed by PlsC, a membrane-bound 1-acylglycerol-3-P acyltransferase that predominately uses acyl-ACP, although some PlsCs also use acyl-CoA. Indeed, FAs from the environment may be converted to acyl-CoA derivatives and be incorporated into the bacteria membrane (Fulco, 1972, Krulwich et al., 1987, Lu et al., 2007).

Bacillus species are characterized by containing three phospholipids as major polar lipids: phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol (Jiang et al., 2014, Choi and Cha, 2014, Yu et al., 2013, Kosowski et al., 2014, Bishop et al., 1967b, Van Pham and Kim, 2014, Lang and Lundgren, 1970, Qiu et al., 2009, Zhai et al., 2012, Seiler et al., 2013). But some aminophospholipids are also found in membranes of some *Bacillus*

strains (Bishop et al., 1967b, Seiler et al., 2013, Wang et al., 2013b, Kang et al., 2013, Choi and Cha, 2014).

2-2 FA catabolism

As previously mentioned FAs are essential components of membranes and are important sources of metabolic energy in all organisms. Consequently, the FA degradation and biosynthesis pathways must be switched on and off according to the availability of FAs to maintain membrane lipid homeostasis. Both intracellular and extracellular supplied FAs are degraded through β -oxidation when the cells are starved of carbon sources. During the β -oxidation cycle, two carbon atoms as an acetyl-CoA molecule is excised from an acyl-CoA molecule at each cycle (see Figure 7) (Matsuoka et al., 2007). After being well studied in *E. coli*, the FA degradation was investigated in *B. subtilis*. This Gram positive bacteria possesses two transcriptional global regulators, FadR (previously named YsiA) that acts to balance the anabolic and catabolic fatty acid pathways (Matsuoka et al., 2007), and FapR (Schujman et al., 2003) that controls the expression of many *fap* regulon members involved in fatty acid and phospholipid metabolism. Their role is described in the next section.

In *B. subtilis*, FadR represses five *fad* (FA degradation) operons, *lcfA-fadR-fadB-etfB-etfA*, *lcfB*, *fadN-fadA-fadE*, *fadH-fadG*, and *fadF-acdA-rpoE*, which encode the enzymes directly involved in β -oxidation (Fujihashi et al., 2014).

LcfA and LcfB most likely encode long-chain fatty acid-CoA ligases which catalyze the transformation of fatty acid into fatty acyl-CoA (Figure 9). FadE likely encodes acyl-CoA dehydrogenase, converting the fatty acyl-CoA into trans-enoyl acyl CoA. This reaction needs one molecule of the coenzyme FAD providing from the action of EtfB and EtfA on FADH₂ (Figure 7). Indeed, EtfA and EtfB likely encode the electron transfer flavoprotein dehydrogenase α - and β -subunits. FadB and FadN likely encode enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase complexes and catalyze the transformation of trans-enoyl acyl CoA into hydroxy-acyl-CoA and the same enzyme converts the last molecule into α -ketoacyl-CoA. Then, FadA (which encodes a 3-ketoacyl-CoA thiolase) transforms α -ketoacyl-CoA into a fatty acyl-CoA which chain is 2 carbons shorter, as a molecule of acetyl is released. A repetitive series of reactions takes place, excising two-carbon units per cycle, until the release of the final molecule of acetyl-CoA.

The role of FadG, FabF, RpoE remains worth investigating for the moment. But it was suggested that FadG was involved in the transport of long chain fatty acids. FadF, a 4Fe-4S ferredoxin protein was considered to be possibly associated with acyl-CoA hydrogenation. RpoE is a RNA polymerase δ -subunit, and was hypothesized to participate in fatty acid metabolism (Matsuoka et al., 2007).

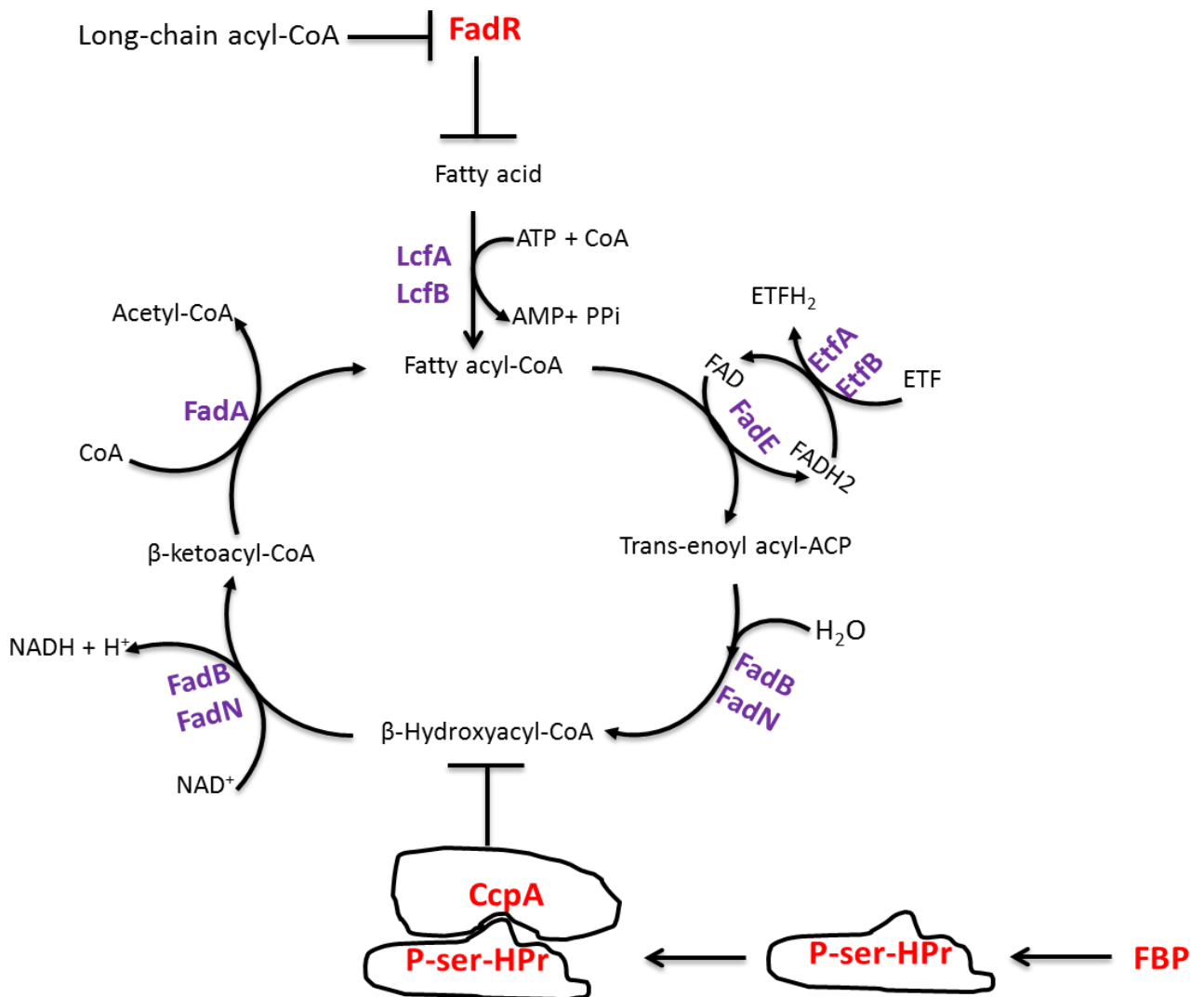


Figure 9: *Bacillus* FA degradation pathway and main regulator. —| indicates a repression. Enzymes are identified in bold and purple and regulators in bold and red.

2-3 Regulation of FA metabolism

A lot of studies revealed that FA biosynthesis is coordinately regulated with phospholipid and macromolecular synthesis and growth in response to changes in the environment. Indeed, the

inhibition of phospholipid synthesis was shown to result in a rapid decrease in the rate of fatty acid synthesis and in the accumulation of acylated-derivatives of ACP (Rock and Jackowski, 1982, Heath and Rock, 1996). FAS are an energetically expensive process explaining why the rate of fatty acid production is tightly regulated, in order to ensure that the supply of membrane phospholipids exactly corresponds to the cell request. Here, we will enumerate some key regulators of the FASII and the FA degradation in *Bacillus* species.

2-3-1 FapR

In *B. subtilis*, the expression of FAS II genes is tightly regulated by the transcriptional regulator, FapR. The gene *fapR* is co-transcribed with *plsX*, *fabD*, *fabG*, *acpP* in a pentacistronic operon named the *fapR* operon (Martinez et al., 2010a). FapR functions as a global transcriptional repressor regulating many genes involved in FA and lipid synthesis (the Fap regulon) with the exception of the ACC protein (Figure 6). (Schujman et al., 2003). The Fap regulon members are *fabHA-fabF*, *fapR-plsX-fabD-fabG*, *fabI*, *fabHB*, *yhfC* and *plsC* with *plsX* and *plsC* involved in phospholipid biosynthesis and FapR was shown to bind to *cis*-sequences in the promoter regions upstream of *fabHA*, *fapR*, *fabI*, *fabHB*, *yhfC* and *plsC* (Schujman et al., 2003). This protein is highly conserved in many Gram-positive organisms, including all species of the *Bacillus*, *Listeria*, *Staphylococcus*, *Clostridium* genera and other related genera (Schujman et al., 2003). Malonyl-CoA as well as malonyl-ACP were shown to regulate directly and specifically the *B. subtilis* FapR (Schujman et al., 2006, Martinez et al., 2010b). Indeed, the binding of whether Malonyl-CoA or malonyl-ACP to FapR, with a similar affinity, operates as an inducer of FapR-regulated promoters by abolishing the repression activity of FapR on these promoters (figure 6) (Martinez et al., 2010b). This mechanism ensures that FA biosynthesis in *B. subtilis* is finely regulated at the transcriptional level by sensing the concentrations of the two first intermediates (malonyl-CoA and malonyl-ACP) in order to balance the production of membrane phospholipids and thus maintain membrane lipid homeostasis.

2-3-2 FadR

In *B. subtilis*, the FA degradation is regulated by FadR, a regulator belonging to the TetR-family of transcriptional regulators. It is an homolog of FabR found in Gram-negative bacteria. FadR represses the FA β -oxidation degradation (Figure 7) by repressing five *fad* operons, *lcfA-fadR-fadB-etfB-etfA*, *lcfB*, *fadN-fadA-fadE*, *fadH-fadG*, and *fadF-acdA-rpoE*, (Fujihashi

et al., 2014, Matsuoka et al., 2007). FadR binds to boxes in the promoter regions upstream these operons. The binding to these boxes is specifically inhibited by the long-chain acyl-CoAs, containing 14 to 20 carbons molecules and not by shorter chain acyl-CoAs (1-10 carbons molecules in the chain) (Fujihashi et al., 2014, Matsuoka et al., 2007). This leads to the de-repression of the *fad* operons and thus to the induction of the FA degradation (Matsuoka et al., 2007).

The β -oxidation of FAs has an crucial function only under certain physiological conditions (Fujita et al., 2007b). For example, during sporulation, the *fadN-A-E* operon was shown to be induced at the onset of sporulation by YvbA involved in cannibalism by sporulating cells (Gonzalez-Pastor et al., 2003). Moreover, the *fadR* operon consisting in *fadR*, *fadB*, *etfB* and *etfA* genes, was shown to be necessary for calcium carbonate biomineralization (Barabesi et al., 2007).

2-3-3 *BkdR*

BkdR is a regulator found in *Bacillus subtilis*, controlling the utilization of isoleucine and valine as sole nitrogen sources (Debarbouille et al., 1999b). Indeed, BkdR positively regulate the *bkd* operon. In *Bacillus subtilis*, this operon consists on the following genes *ptb*, *bcd*, *buk*, *lpdV*, *bkdAA*, *bkdAB* and *bkdB*. These genes encode the enzymes that catalyze the degradation of branched-chain amino acids into precursors of anteiso-branched FA.

The expression of the *bkd* operon was shown to be induced by the presence of isoleucine or valine in the growth medium and depended upon the presence of the sigma factor SigL (Qi et al., 2008) and is also inducible by a temperature downshift from 37 to 18 degrees C in *B. subtilis* (Nickel et al., 2004). SigL as well as *bkdR*, were showed to participate in the *B. subtilis* cold-shock adaptation. (Wiegeshoff et al., 2006)

2-3-4 *ComA*

ComA, belonging to the major quorum response Com signalling pathway in *B. subtilis*, was shown to regulate both directly and indirectly the expression of genes involved in FA metabolism, including FapR (Comella and Grossman, 2005). ComX–ComP–ComA pathway controls quorum responses, the peptides of this pathway are secreted into the environment and elicit a regulatory response at a given concentration in order to contribute to the induction of competence development, sporulation, degradative enzyme production and antibiotic production (Comella and Grossman, 2005, Grossman, 1995, Msadek, 1999, Tortosa and

Dubnau, 1999). A quorum response is mediated by a secreted 10-amino-acid modified peptide, ComX pheromone which activates ComP, a receptor histidine kinase, which then activates ComA, a response regulator transcription factor. The expression of the FapR regulon, including the *fapR plsX fabDG* operon, *yhdO*, *fabHB*, the *fabHAF* operon, and *fabI*, was reduced in a *comA* null mutant (Comella and Grossman, 2005). As adjustment of the lipid composition of membranes is important for growth and gene regulation under a variety of different conditions, Comella *et al.* hypothesize that during the quorum response, changes in lipid composition of the membrane might be important for accommodating the wide variety of developmental changes that might occur during competence, sporulation and biofilm formation (Comella and Grossman, 2005).

2-3-5 CcpA

The catabolic control protein CcpA was described to repress the five operons belonging to the FadR regulon including the *fadR* operon (Tojo *et al.*, 2011). Activated CcpA represses many catabolic genes and operons, mainly those involved in carbon, nitrogen, and phosphate metabolism. In *B. subtilis*, this repression occurs after the formation of complex of CcpA with the seryl-phosphorylated form of a histidine-containing protein (P-Ser-HPr) or its homologue (P-Ser-Crh, Crh meaning Catabolic repression Hpr) and the binding of this complex to catabolite-responsive elements (CRE) of target operons (Miwa *et al.*, 2000, Galinier *et al.*, 1998).

2-3-6 Sigma W

Several extracellular function (ECF) σ factors of *B. subtilis* are involved in stress responses elicited by compounds that affect membrane integrity and/or fluidity.

The ECF sigW was recently described to respond to compounds that increase membrane fluidity (Kingston *et al.*, 2011). The binding of SigW to the SigW-dependent promoter within the *fabHa-fabF* operon down-regulates *fabHA* but up-regulates *fabF* leading to a higher proportion of straight chain FA and a longer average chain length for membrane phospholipids. This results in a membrane having a reduced bilayer fluidity and an increased resistance to detergents and antimicrobial compounds produced by other *Bacillus* species (Kingston *et al.*, 2011)

2-3-7 two component system regulation

Some two component systems were described for their role as thermosensor to maintain the membrane homeostasis.

B. subtilis DesK/R was the first two-component system (TCS) described as involved in the cold adaptation of a *Bacillus* strain (Aguilar et al., 2001b). DesK/R is responsible for maintaining the membrane fluidity for low-temperature adaptation. The sensor protein DesK is a multipass transmembrane histidine-kinase that senses an increase in membrane thickness when the *B. subtilis* growth temperature decreases (Aguilar et al., 2001b, Cybulski et al., 2010b, Inda et al., 2014a). Then, its cognate response regulator DesR regulates the expression of the desaturase *des*, in charge of increasing the proportion of the membrane UFAs and thus maintain an optimal membrane fluidity (Aguilar et al., 2001b). DesK/R was further proposed to also be effective whether at constant temperature or after a temperature downshift when a decrease in membrane fluidity is sensed (Cybulski et al., 2002).

CasK/R was recently described as a TCS involved in *B. cereus* cold adaptation (Diomandé et al., 2014). In contrast to the membrane protein DesK, CasK is supposed to have a cytoplasmic location (Diomandé et al., 2014). Recent evidences showed that this TCS regulates genes involved in both FA synthesis and FA degradation (Diomandé et al, unpublished data), including the two genes encoding desaturases *desA* and *desB*. These data demonstrated that during growth at low temperature CasK/R regulates the membrane FA composition and mainly the UFAs synthesis via the regulation of *desA*. DesA is a $\Delta 5$ desaturase, homolog to the *B. subtilis* Des protein

Several studies identified in *Bacillus* species, the $\Delta 5$ desaturase as involved in low temperature adaptation (Fulco, 1967, Fulco, 1972, Fulco, 1969, Kaneda, 1972, Bredeston et al., 2011). The two examples of DesK/R and CasK/R illustrate that the regulation of membrane fluidity at low temperature may involve two component systems in *Bacillus* species.

2-3-8 Spo0A

Spo0A represents a master transcription factor of *Bacillus* strains as Spo0A regulon comprises more than 500 genes (Piggot and Hilbert, 2004). This protein plays a major role in the formation of *Bacillus* spores. Sporulation is triggered by starvation-related signals that activate Spo0A by phosphorylation. During the initial developmental steps of the forespore, the formation of an asymmetrically positioned septum delimited by two membranes of

opposite topology creates a small and specifically localized cell, the forespore, and a larger cell, called the mother cell (de Hoon et al., 2010). As previously mentioned an active and robust *de novo* FA and membrane lipid synthesis occurs during sporulation (Pedrido et al., 2013, Schujman et al., 1998). Spo0A controls the *accDA* operon involved in malonyl-CoA synthesis (Pedrido et al., 2013). Through this regulation, Spo0A consequently regulates the FASII, because as previously enounced malonyl-CoA is a direct and specific negative modulator of FapR (Pedrido et al., 2013).

2-3-9 CodY

CodY is a global transcriptional regulatory originally identified in *B. subtilis* (Slack et al., 1995). Its role was however defined in other *Bacillus* strains (Hsueh et al., 2008, van Schaik et al., 2009). The binding of CodY to DNA is potentiated by two classes of effector molecules that act as information input signals the branched-chain amino acids (BCAAs) comprising isoleucine, leucine, and valine and the nucleoside triphosphate GTP (Shivers and Sonenshein, 2004). A recent study showed that CodY not only responds to these metabolites but also regulates the genes that direct their synthesis (Brinsmade et al., 2010).

Because, a lack of control of the BCAAs may be responsible for the growth defect in the BCAAs, BCAAs biosynthesis have to be controlled at the transcriptional, posttranscriptional, and enzymatic levels to ensure that appropriate amounts of precursors are available for the synthesis of proteins, branched-chain FAs, and coenzyme A (Brinsmade et al., 2010). Therefore, CodY regulates expression of the genes required for BCAAs synthesis by binding to conserved DNA sequences (Shivers and Sonenshein, 2005). This consequently regulates the branched FAs pathway as BCAAs are the precursors of this pathway.

Moreover CodY was described to negatively regulate the *bkd* operon involved in the isoleucine and valine degradation (Debarbouille et al., 1999a).

In conclusion to this section, it clearly appeared that the *Bacillus* FASII and FA degradation comprises key enzymes displaying each a key role for membrane integrity. Disturbing the function of one of them may affect the membrane homeostasis. The number of regulators of FA metabolism seems to be as numerous as the number of adaptation mediated by FA composition changes is. They included repressors, sigma factors, antibiotics, TCS, and others regulator proteins.

3- Exogenous Fatty acids: their impact on *Bacillus* growth ability

Food industry is often aiming at producing minimally processed food preserved from pathogens. New natural antimicrobial compounds from different origins can be used, including FAs, because of two main particularities: their lipophilicity and their acidity. Several investigations were done in order to better understand the anti-microbial ability of exogenous FAs. Some examples are discussed below.

3-1 Inhibition of spore's germination

One antimicrobial activity of exogenous FAs resides in their capacity to inhibit spores germination and/or outgrowth. Indeed, in presence of the lauric acid (12C) and oleic acid (18C), *B. cereus* spore germination is totally inhibited (Ababouch et al., 1994). They hypothesized that this was the consequence of the inhibition of germinant binding to germination sites. This inhibition was reversible as no more inhibition was observed when the medium was freed of these FAs.

Moreover, even on fully germinated spores, an inhibition of the outgrowth was observed in presence of lauric acid and two polyunsaturated FAs with 18C, linoleic and linolenic acids (Ababouch et al., 1994). The underlying mechanisms depended on the type of FAs but the inner membrane of the germinated spores was shown to be the site of action of these inhibitors. Acid sorbic, a short UFA, was also shown to delay the germination and the outgrowth of *B. cereus* spores (van Melis et al., 2011) but it also inhibits spore germination (Smoot and Pierson, 1981).

This allowed to identify two potential types of FAs: (i) FA preserving foods containing spores; (ii) FAs stabilizing food, contaminated with germinated spores and vegetative cells, from outgrowth and multiplication. The combination of these two types of FAs guarantees a food in which dormant, germinated spores and vegetative cells are controlled.

Some FAs like laurate, palmitate, stearate and or some FA esters inhibit *Bacillus* spores in varied culture conditions characterized by different T°, pressure and when combined with other chemical compounds such as sucrose or monoglycerol (Klangpetch et al., 2013, Shearer et al., 2000, Feijoo et al., 1997). But, for most of the cases cited above, an inhibitory effect was observed rather than a lethal effect on the *Bacillus* spores.

However, studies also described these FAs, more specifically the UFAs, for their ability to highly decrease bacterial spore heat resistance leading to a lethal effect to spores. A model describes the decrease in D-values (time for a log population decrease) in the presence of free fatty acids during low sterilization treatment (Lekogo et al., 2010).

3-2 Growth inhibition

Exogenous FAs not only inhibit spores but also vegetative cells properties. Lee *et al.* demonstrated that linolenic acid, in presence or not of monoglyceride displayed strong antimicrobial activity on *B. cereus* cells (Lee et al., 2002a). The addition of linolenic acid in the media was accompanied by a drastic increase of the bacterial extracellular ATP concentration and a decrease of the intracellular ATP concentration (Lee et al., 2002a). Palmitic and stearic acid from cold pressed clove oil also inhibited growth of a range of microorganisms including *B. subtilis* (Assiri and Hassanien, 2013).

The growth of the protonophore-resistant strain C8 of *B. megaterium*, in the presence of oleic acid highly reduced its resistance to low concentrations of the carbonylcyanide *m*-chlorophenylhydrazone (CCCP) protonophore (Clejan et al., 1988). This loss of resistance was explained by the higher level of UFAs in the membrane of this bacterium when grown in presence of oleic acid. Conversely, the growth of the CCCP-sensitive wild-type strain in the presence of a SFA, stearic acid, increased the resistance to protonophore by increasing the saturated /unsaturated FA ratio (Clejan et al., 1988).

Thus, the effect of a given FA on growth seems to depend on both its properties and the environmental conditions.

3-3 Improved growth

In contrast, exogenous FA may act as growth activator for *Bacillus* vegetative cells in particular conditions. As described above, *B. megaterium* is able to incorporate free FAs from the media and a modulation of the resistance to the protonophores may be observed and depending on the FA. Stearic acid (Clejan et al., 1988) in *B. megaterium* as well as palmitic acid in *B. subtilis* (Krulwich et al., 1987) are SFAs able to improve the resistance to protonophore.

In contrast to *B. subtilis* and *B. megaterium* (Fujita et al., 2007a), *B. cereus*, do not seem to be able to use free FA from the external environment (De sarrau & Nguyen-thé, personal communication). But this bacterium is able to use exogenous phospholipids and integrate the FAs present in these phospholipids into its membrane (de Sarrau et al., 2013c). For example, at low temperature under anaerobiosis, growth of *B. cereus* strains is presumably inhibited because membrane lipids and fluidity cannot be adapted to low temperatures without oxygen (de Sarrau et al., 2012b). It was shown that UFAs from food (spinach) or from the media of the bacteria could be incorporated into membrane to allow a better adaptation of strains grown at low temperature under anaerobiosis. Indeed, under this condition and in presence of precursors of UFAs, the growth of this strains was similar to the growth in aerobiosis at low temperature (de Sarrau et al., 2013c).

In *B. cereus*, an exogenous source of UFAs was shown to improve the growth ability of the *casK/R* mutant (Diomandé et al., chapter 3). This mutant displayed a decreased production of UFAs during growth at low temperature and this exogenous source of UFAs allowed supporting the membrane-level modifications caused by the mutation of the *casK/R*.

Some species such as *B. megaterium* may develop in medium supplemented with exogenous FAs from their growth medium, oxidize them and produce polyunsaturated FAs that are important industrial materials for manufacturing value-added products such as oxygenated oils (Hou, 2009).

Conclusion

Fatty acids are a universal pillar component of membrane cell. Extensively studied in *Bacillus* species, their involvement in a wide type of adaptation highlights their predominant role for vegetative cells survival, growth and spore resistance. In *Bacillus* species, FAs represent a good biomarker to determine the exact repartition among *Bacillus* species depending on their environmental niches. At metabolic level, FASII is connecting to the major cell metabolisms and this FA synthesis appears to be finely regulated depending on the cell need. Numerous regulators control the FA metabolism and the regulation is clearly dependent on the cell environmental conditions. *Bacillus* strains are also able to use FAs from their environment, including from food. This is consistent with the fact that some *Bacillus* strains represent major cause of FBI. But, the impact of the use of these exogenous FA may be positive or negative

depending on the *Bacillus* cell state (spores or vegetative cells) and it seems to depend on the environmental conditions and on the type of FAs. The antimicrobial activity of some of these exogenous FAs seems to be a good alternative for food preservation during processing and conservation of food.

III) Les systèmes à deux composants, un autre mécanisme d'adaptation bactérien

1) Un mécanisme d'adaptation universel

Bien connus chez les eucaryotes, les transductions de signaux chez les bactéries, par le moyen de la phosphorylation, ont été découvertes voilà plus de 30 ans à la fin des années 70 (Wang and Koshland, 1981). Chez les bactéries ainsi que les archées, les systèmes à deux composants (TCS) représentent les voies de signalisations principales permettant à la bactérie de répondre à des signaux environnementaux donnés (Galperin, 2005b). Un système de transduction à deux composants est un module ubiquitaire composé d'une histidine kinase (HK) et d'un régulateur de réponse (RR), détectant et répondant à des changements environnementaux spécifiques (Kojetin et al., 2007). L'analyse de génomes entiers et de méta-génomes a permis de mettre en évidence que les domaines des systèmes à deux composants représentent le 2^e type de domaines Pfam les plus rencontrés chez les bactéries juste après les domaines des ABC-transporteurs (Bateman et al., 2004, Whitworth and Cock, 2008, Whitworth and Cock, 2009).

Les TCS sont impliqués dans l'adaptation à de nombreuses conditions environnementales telles que, les milieux acides (Cui et al., 2012), l'anaérobiose (Hartig and Jahn, 2012), les milieux pauvres en nutriments (Sun et al., 1996), les températures froides (Aguilar et al., 2001b), la présence d'antibiotiques (Jordan et al., 2008). Ils contrôlent aussi la virulence et les interactions avec l'hôte (Song et al., 2012b, Mike et al., 2014, Esbelin et al., 2009). Ils contrôlent beaucoup des comportements bactériens tels que la mobilité (Boll and Hendrixson, 2013), le chimiotactisme (Lyon and Novick, 2004), la sporulation (Jiang et al., 2000), la formation de biofilm (Moraes et al., 2014) et le quorum sensing (Cao et al., 2014).

2) Fonctionnement classique des TCS

Classiquement, l'HK et le RR possèdent chacun deux domaines. L'HK porte à son extrémité N-terminal, souvent un domaine senseur associé à la membrane et à son extrémité C-terminal, un domaine auto-kinase et de transfert de phosphate. Le RR possède quant à lui un domaine receveur de phosphate à son extrémité N-terminal et en C-terminal, un "output domain", très majoritairement un domaine de fixation à l'ADN pour la régulation de gènes d'intérêt. Les principaux domaines retrouvés chez les TCS sont détaillés dans le paragraphe suivant. *In*

in vivo, chacune de ces protéines fonctionne en homodimères (Yamada and Shiro, 2008, Wolanin et al., 2002).

Le principe de fonctionnement des TCS est le même pour tous, il repose sur un transfert de groupement phosphate entre l'HK et le RR (Figure 10). Lorsqu'un stimulus signalant un stress environnemental est perçu par l'HK au niveau du domaine senseur, la protéine s'auto-phosphoryle par hydrolyse d'une molécule d'ATP, grâce à son domaine kinase, au niveau d'un résidu histidine conservé (Cheung and Hendrickson, 2010b, Dutta et al., 1999). Puisque l'HK est active sous la forme d'un dimère, l'autophosphorylation mentionnée ici implique un transfert croisé du phosphate du domaine kinase d'une unité de dimère vers un résidu histidine de la deuxième unité du dimère (de Been, 2009). Le groupement phosphate est alors transféré à un résidu aspartate conservé sur le RR, au niveau N-terminal. Celui-ci est alors activé et sa conformation change, lui permettant alors, comme un facteur de transcription, de se fixer au niveau de régions promotrices grâce à son domaine en C-terminal et de réguler l'expression de gènes impliqués dans la réponse adaptative au stress environnemental (Yamada and Shiro, 2008, Robinson et al., 2000).

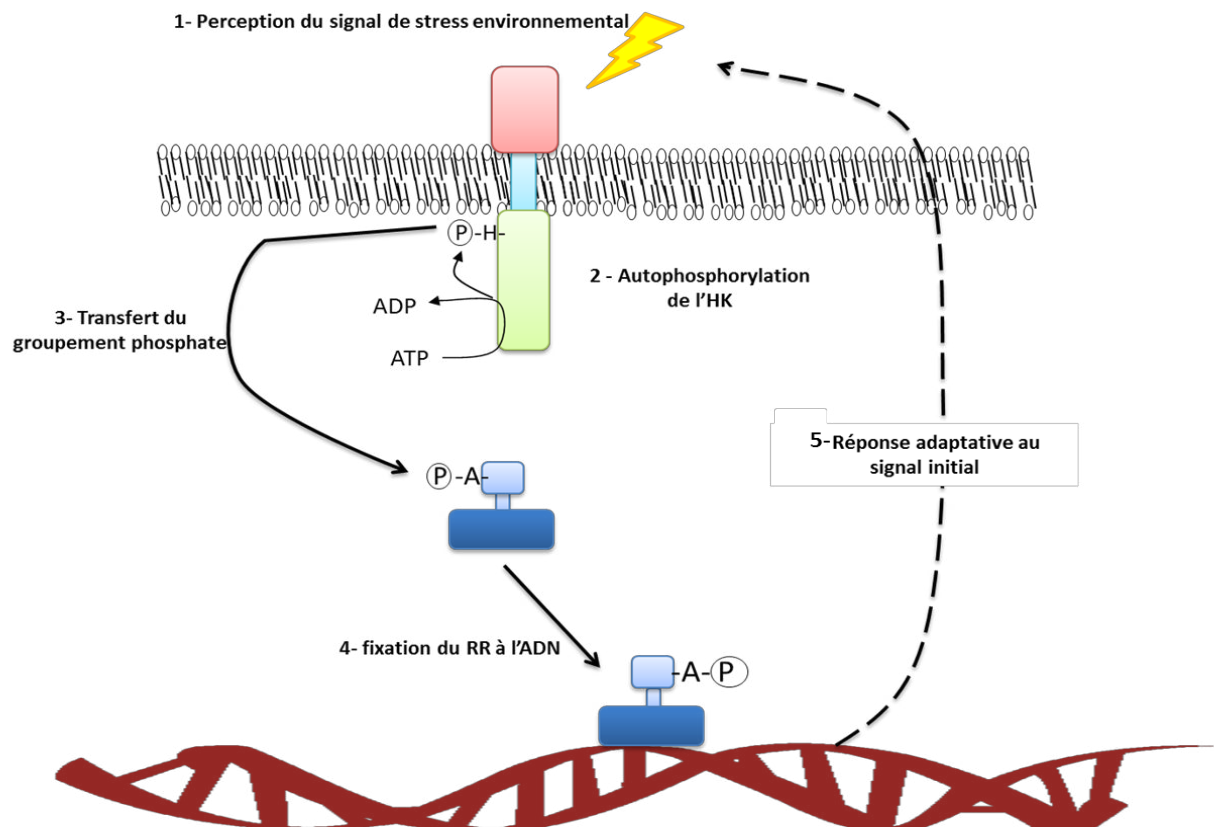


Figure 10 : Transduction de signal classique d'un TCS lors de la perception d'un signal. Dans un but illustratif, les protéines ont été représentées, dans cette figure, sous la forme de des monomères de protéines

3) Domaines protéiques de TCS

Onze familles de TCS ont été identifiées suite à des analyses phylogénétiques sur la base des domaines rencontrés chez les HKs et les RRs (Koretke et al., 2000, Grebe and Stock, 1999, Mizuno, 1997a). Ces onze familles de TCS auraient évolué différemment à partir d'un TCS ancêtre commun (Koretke et al., 2000). Les deux familles les plus fréquemment retrouvées sont la famille 1a (OmpR) et la famille 7 (NarL) (Grebe and Stock, 1999, de Been et al., 2006a). Les protéines formant un TCS sont constituées d'un nombre variable de domaines.

Comme précédemment mentionné, les TCS sont généralement décrits comme étant constitués d'une histidine kinase constituée de 2 domaines fonctionnels, un domaine senseur et un domaine transmetteur de phosphate (Figure 11) et un régulateur de réponse avec un domaine receveur de phosphate et domaine de réponse ou de régulation. L'HK étant généralement membranaire, plusieurs cas de figure sont possibles quant à l'agencement des domaines (Figure 11).

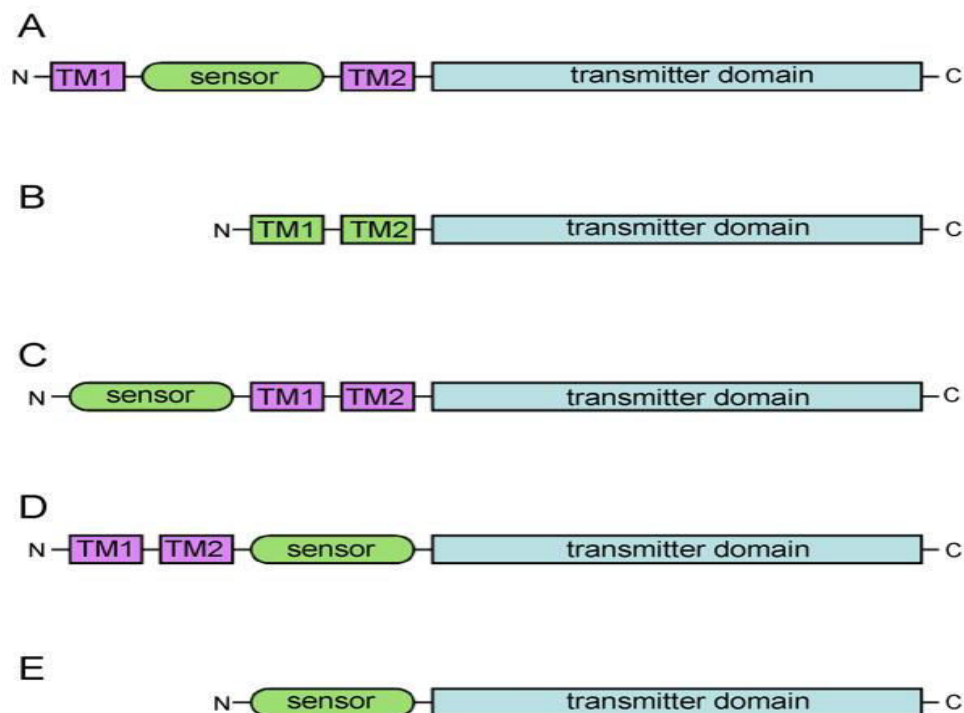


Figure 11 : Organisation des domaines des HKs (Cheung and Hendrickson, 2009)(Cheung and Hendrickson, 2010a). En A, B, C et D sont représentés les agencements de domaines possibles pour des protéines transmembranaires. En E, la représentation d'une histidine kinase cytosoluble donc sans domaine transmembranaire. Il existe aussi des HKs sans domaine senseur comme représenté en B.

TM1 et TM2 = domaines transmembranaires ; sensor = domaine senseur de l'HK ; transmitter domain = domaine de transmission de phosphate

Le transfert de phosphate entre l'HK et le RR est un mécanisme important dans la réponse adaptative. Un domaine par protéine constituant le TCS y jouerait un rôle (Williams and Whitworth, 2010) et plus précisément, en moyenne 1,16 domaine de TCS serait impliqué dans ce transfert (Williams and Whitworth, 2010) (tableau 4).

Tableau 4 : Nombre de domaines de transfert de phosphate par protéine constituant le TCS (Robert William et al., 2010) (Williams and Whitworth, 2010)

| TCS domains | 1 | 2 | 3 | 4 | 5 | 6 | 7+ | Total |
|--------------------|----------|----------|----------|----------|----------|----------|-----------|--------------|
| # Proteins | 38317 | 3605 | 1136 | 343 | 20 | 2 | 3 | 43426 |

En allant plus loin sur cette analyse, on observe que lorsqu'il n'y a qu'un seul domaine de transfert de phosphate par protéine de TCS, il s'agit plus fréquemment de domaine receveur donc de domaine présent sur le RR (Tableau 5) que de domaine transmetteur, présent sur l'HK (Williams and Whitworth, 2010).

Tableau 5 : Nombre de domaines receveur (R) ou transmetteur (T) de phosphate observés chez les TCS (Robert William et al., 2010)

| Domains | 0R | 1R | 2R | 3R | 4R | 5R | 6R | Total |
|----------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|--------------|
| 0T | 1014 | 21743 | 301 | 14 | 0 | 0 | 0 | 23072 |
| 1T | 15702 | 3919 | 588 | 88 | 4 | 0 | 0 | 20301 |
| 2T | 2 | 33 | 13 | 4 | 0 | 0 | 1 | 53 |
| Total | 16718 | 25695 | 902 | 106 | 4 | 0 | 1 | 43426 |

Il existe des RR possédant un unique domaine, le domaine récepteur de phosphate. En effet, ces protéines sont très généralement des intermédiaires d'interactions protéiques (Jenal and Galperin, 2009). Elles vont avoir pour rôle de relayer le groupement phosphate entre plusieurs TCS (figure 12). Ce sont donc des connections entre TCS facilitant les communications croisées, en feedback ou à longue portée dans un réseau de phosphorylation de TCS.

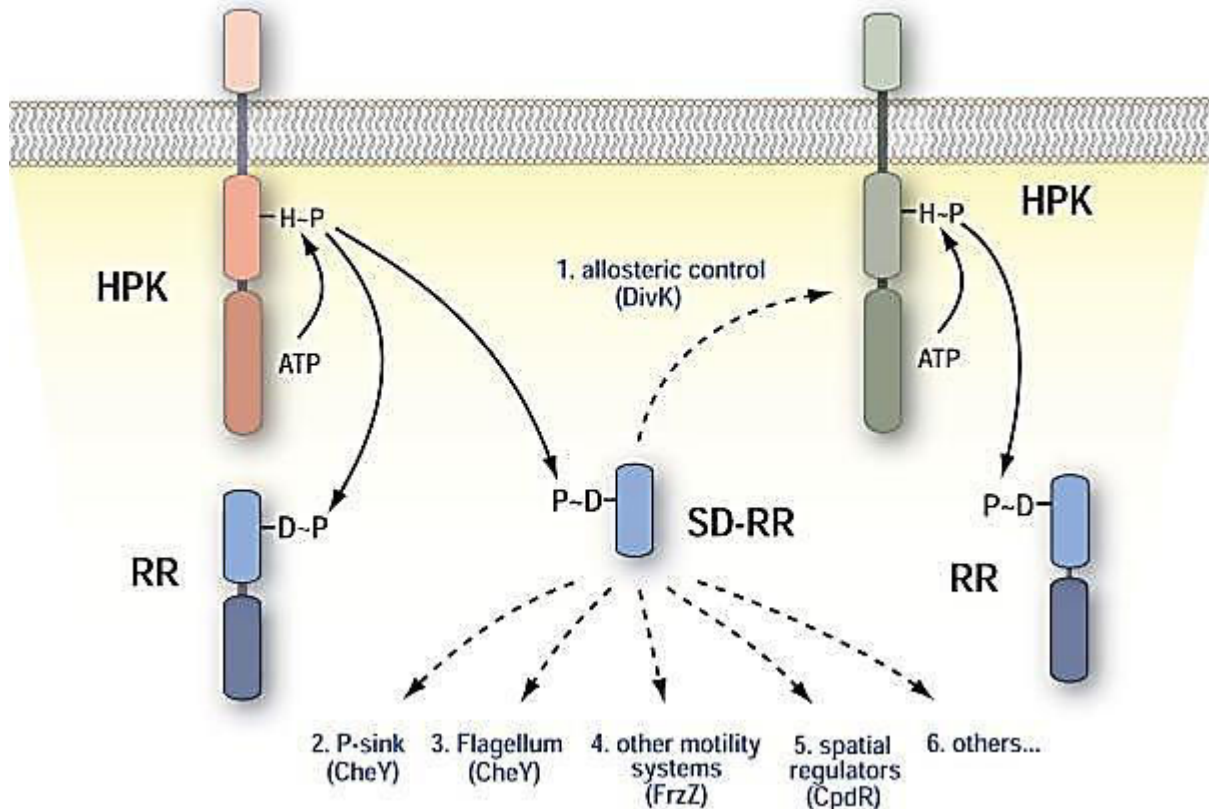


Figure 12: Fonction des RR à un domaine (SD-RR). Les domaines des HK (HPK sur le schéma), RR et SD-RR (RR à un domaine) sont représentés schématiquement. Les lignes continues indiquent les réactions de phosphorylation et les lignes en pointillés résumant les mécanismes de régulations impliquant les SD-RR (Jenal *et al.*, 2009)(Jenal and Galperin, 2009).

4) Le phosphorelais

En plus du mode de transfert classique de phosphate entre les HKs et les RRs d'un même TCS, comme décrit précédemment et représenté dans la figure 13, il existe aussi le phosphorelais. Un phosphorelais est une succession de transfert de phosphate ayant toujours comme origine l'HK mais incluant des intermédiaires dans le transfert de phosphate jusqu'au RR partenaire de l'HK. Deux intermédiaires sont souvent retrouvés, un RR intermédiaire ne possédant pas de domaine effecteur (Appleby *et al.*, 1996, Mitrophanov and Groisman, 2008, Perraud *et al.*, 1999) et une protéine de transfert de phosphate possédant une histidine (Mitrophanov and Groisman, 2008). Dans certains phosphorelais, ces deux dernières protéines sont fusionnées à l'HK en un seul polypeptide (Mitrophanov and Groisman, 2008). Le phosphorelais est rencontré par exemple lors du signal de transduction pour la sporulation depuis l'HK KinA jusqu'au RR final Spo0A chez *B. subtilis* (Wang *et al.*, 1997).

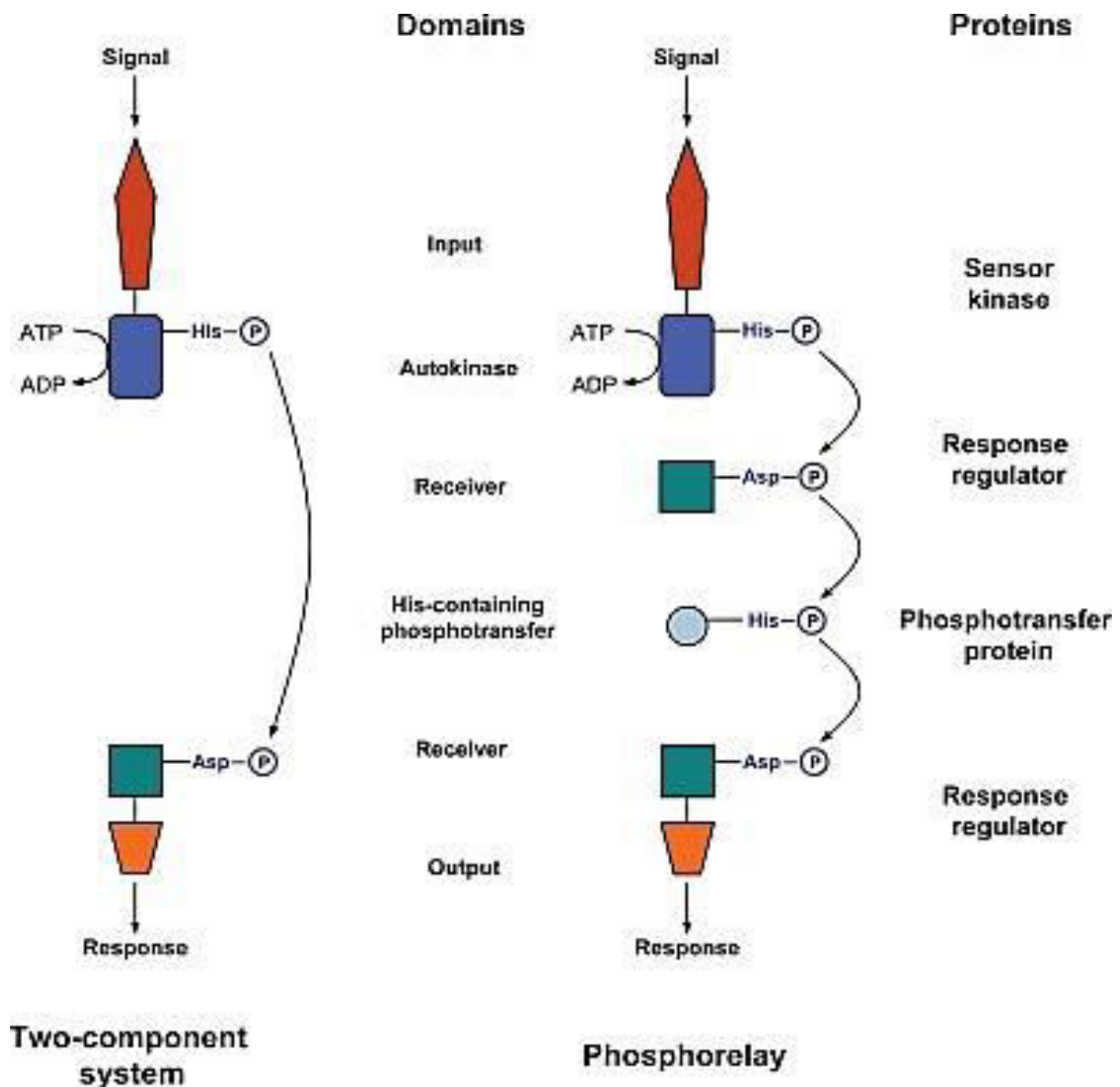


Figure 13 : schéma représentant les protéines du TCS et leur domaine dans un fonctionnement à deux composants (gauche) et dans un mécanisme de phosphorelais (droite) (Mitrophanov *et al.*, 2008) (Mitrophanov and Groisman, 2008).

5) Organisation génétique des TCS

Il a été proposé un "cut-off" pour définir qu'un gène codant pour une HK et celui codant un RR sont appariés c'est-à-dire qu'ils fonctionnent ensemble dans un même TCS. En effet, la distance inter-génique entre le gène codant pour l'HK et celui codant pour le RR a été estimée à 200 pb et représente un intervalle *in cis* c'est-à-dire sur le même brin ou *in trans* (lorsque les gènes sont sur deux brins différents) (Williams and Whitworth, 2010). Cela sous-entend qu'en dessous d'une distance intergénique de 200 pb entre ces gènes on peut dire que ces gènes sont co-localisés pour des raisons fonctionnelles. Si la distance inter-génique est

supérieure à 200pb alors leur affiliation fonctionnelle en un même TCS est arrivée par hasard. Ainsi, une HK ou un RR sera considéré systématiquement comme « orphelin(e) » lorsque le gène codant pour une de ces protéines est à une distance supérieure à 200 pb respectivement d'un gène codant pour un RR ou une HK.

Pour l'étude de Williams *et al.* (Williams and Whitworth, 2010), 29,556 régions inter-génique en *cis* et 13,086 régions inter-génique en *trans* ont été testées, celles-ci avaient été obtenues à partir d'une base de données regroupant les 44,008 gènes codant pour les TCS de tous les génomes procaryotes séquencés, le P2CS (Barakat *et al.*, 2011). Un quart de régions inter-géniques étudiées avait une valeur inférieure à 0pb suggérant donc que ces gènes appariés se superposent formant ainsi une kinase hybride c'est-à-dire une fusion HK-RR. Ce phénomène est un phénomène fréquent parmi les TCS (Cock and Whitworth, 2007a) et dans le génome des procaryotes (Palleja *et al.*, 2008).

Des exemples de kinase hybrides sont représentés dans la Figure 14 (Cock and Whitworth). Un exemple de kinase hybride membranaire et une hybride kinase cytoplasmique y sont représentés. Dans ces cas, il arrive qu'il y ait des systèmes réduits au minimum, par exemple uniquement à un domaine transmetteur de phosphate et un domaine receveur de phosphate (Figure 14).

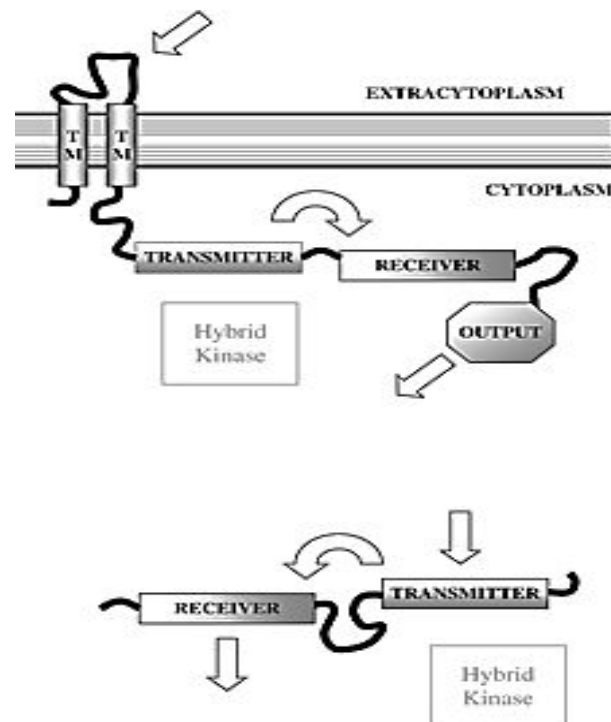


Figure 14 : Exemple de kinase hybride avec un domaine « output », c'est-à-dire effecteur (Schema du haut). Une kinase hybride sans le domaine de détection de signal et le domaine

de détection est représenté dans le schéma du bas. Les flèches représentent le chemin de transfert d'information entre les domaines du TCS (Cock and Whitworth).

6) L'activité phosphatase des HK

La plupart des histidines kinases sont bi-fonctionnelles car en plus de transférer le groupement phosphate au RR, elles peuvent aussi agir comme phosphatases, déphosphorylant ainsi leur partenaire (Huynh and Stewart, 2011) (Figure 15). Cette réaction module le niveau de régulation du RR et inhibe aussi les voies de signalisation après que la réponse adaptative soit effective (Huynh and Stewart, 2011). De plus, l'activité de phosphatase de l'HK permet de limiter des phosphorylations non désirées du RR partenaire par d'autres kinases ou petites molécules donneuses de groupement phosphate (Kenney, 2010). Par exemple, un grand nombre de RR peut être phosphorylé de manière non spécifique en fonction du pool cellulaire d'acetyl-phosphate (McCleary et al., 1993, Klein et al., 2007, Boll and Hendrixson, 2011). Ce genre d'activation du RR par phosphorylation indésirable peut être évité grâce à l'activité phosphatase de l'HK (Siryaporn and Goulian, 2008). Ces phosphorylations indésirables peuvent ainsi, lorsque le gène codant pour une HK est mutée, entraîner une activation indésirable de son RR partenaire dans des conditions où celui-ci ne devrait pas être induit (Siryaporn and Goulian, 2008).

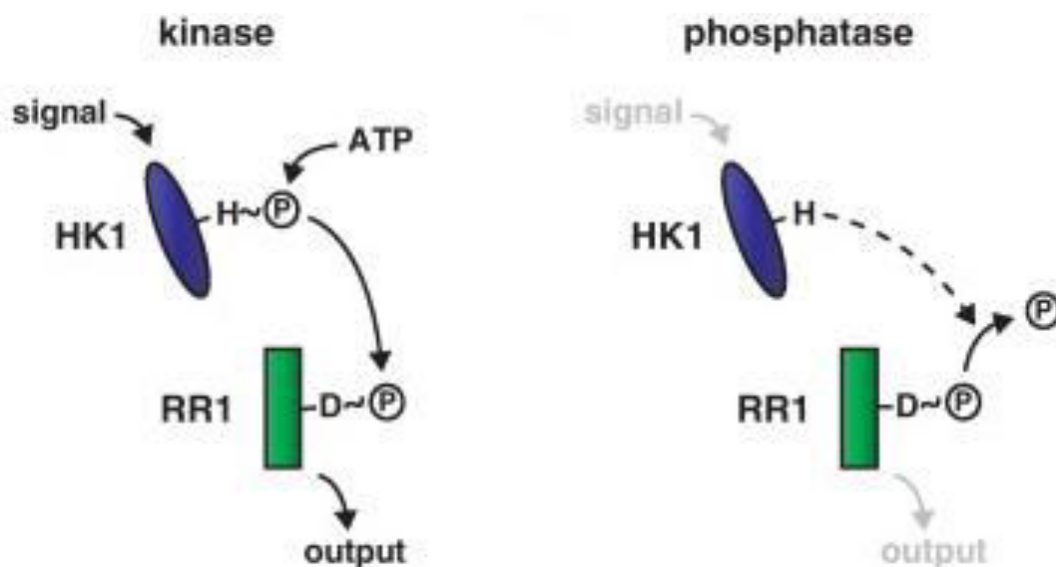


Figure 15: les deux activités des HK, kinase en A et phosphatase en B (Podgornaia *et al.* , 2013 (Podgornaia and Laub, 2013b))

7) Les TCS totalement cytoplasmiques

Tandis que les régulateurs de réponse (RR) sont, de par leur fonction, quasi-obligatoirement cytoplasmiques, les histidines kinases (HK) décrits dans la littérature scientifique sont souvent membranaires. Selon les études de Cock et Whitworth, 88% de HK possèdent des domaines transmembranaires (Cock and Whitworth, 2007b), tandis qu'Ulrich et ses collègues estiment cette proportion à 73% (Ulrich et al., 2005). Ainsi, il existe aussi des HK entièrement cytoplasmiques.

Trois domaines senseurs majeurs caractérisent les HK cytoplasmiques: les domaines PAS, domaines GAF et domaines PHY.

La plupart des HK cytoplasmiques possèdent un domaine PAS (Cheung and Hendrickson, 2010a). Un des exemples les plus intéressants de HK à domaine PAS est celui de la kinase A (KinA) contrôlant l'initiation de la sporulation de *Bacillus subtilis* (Krell et al., 2009a). Cette protéine possède trois domaines PAS. Deux d'entre eux, PAS B et PAS C reconnaissent le signal primaire. Le dernier a la capacité de détecter et hydrolyser l'ATP, ce qui est original pour un domaine senseur de HK, ces fonctions étant généralement réalisées par le domaine "transmetteur" des HK. Stephenson et Hoch ont suggéré que cette fonction permet à la protéine d'apprécier l'état énergétique de la cellule (Stephenson and Hoch, 2001).

Le domaine PHY est souvent retrouvé chez des protéines nommées phytochromes. Ces molécules sont des capteurs de lumière, récemment découverts chez les bactéries et, habituellement présents chez les bactéries photosynthétiques. L'HK RPBphP4 de *Rhodospseudomonas palustris* est un bon exemple. Plus précisément, l'achromo-forme de cette protéine, une forme non liée à la biliverdine, pigment tétrapyrrolique vert de la bile. La protéine a trois domaines senseurs : PAS, GAF et domaine PHY. Le domaine PHY dans ce cas, capte le signal lumineux et perçoit le changement de l'état redox (Krell et al., 2009a).

Le troisième type de domaine, le domaine GAF, a été initialement identifié dans des phosphodiésterases régulées par la cGMP. Une bonne illustration de HK à domaine GAF est celle des protéines DOS S et DOS T de *Mycobacterium tuberculosis*. Ces deux HKs possèdent chacune un domaine senseur GAF contenant de l'hème à activité redox et sont capables de se lier à des ligands diatomiques comme O₂, NO et CO (Vos et al., 2012). Elles activent le même régulateur transcriptionnel DOS R qui joue un rôle crucial dans la virulence

de l'agent pathogène (Vos et al., 2012). Cependant d'autres HKs à domaines GAF sont capables de reconnaître tout simplement des c-GMPc (Ho et al., 2000b).

Il existe une similitude frappante dans les structures des domaines GAF et PAS. Selon Ho et al. cela révèle une claire similitude d'évolution entre ces deux domaines (De Maayer et al., 2014). De plus, le fait que ces domaines, pris séparément, soient présents dans un grand nombre de protéines impliquées dans les voies de signalisation et détection, semble confirmer cette hypothèse. Les domaines GAF sont aussi retrouvés dans un grand nombre de phytochromes. (Ho et al., 2000b, Kehoe and Grossman, 1996).

Une autre particularité des HK cytosoliques est liée au fait que ces protéines sont fréquemment la cible de protéines régulatrices cellulaires qui modulent leur activité autokinase (Krell et al., 2009a). En outre, la duplication du domaine senseur est très fréquemment observée pour les HK cytosolique. Des études montrent qu'une HK cytosolique avec un domaine senseur unique semble très rare (Mascher et al., 2006b). Le phénomène de duplication est particulièrement prononcé chez les myxobactéries, qui ont un mode de vie complexe. Ainsi, il semble que la duplication du domaine senseur soit due à la nécessité de la cellule d'acquérir la capacité de reconnaître plusieurs signaux. Krell *et al.* ont donc émis l'hypothèse que cette capacité pourrait être un avantage évolutif des HK cytosoliques par rapport à celles qui sont transmembranaires (Krell et al., 2009a).

Huit protéines cytosoliques sont décrites chez la souche *B. cereus* ATCC 14579. Quatre d'entre elles sont putativement identifiées comme ayant un rôle dans la sporulation (BC_2243, BC_1428, BC_2619 et BC_5455), une dans le chimiotactisme (BC_1628), une dans le catabolisme des acides gras à courtes chaînes (BC_3641), deux ont une fonction inconnue (BC_5412 et BC_2216) (Ivanova et al., 2003, Abee et al., 2011a). L'une de ces histidines kinase, BC_2216, constitue le cœur du projet de mon projet de thèse.

8) TCS chez *B. cereus* *sl.*

Les souches de *B. cereus sensu lato* possèdent une cinquantaine de HK et de RR contre une trentaine chez *B. subtilis* (de Been et al., 2006a). Une quarantaine de TCS environ sont décrits comme appariés, fonctionnant donc comme partenaires. En outre, certains HK et RR sont fusionnés en une seule protéine formant donc une kinase hybride. Chez les souches de *B. cereus* analysées, deux hybrides kinases sont retrouvés contre seulement un chez les autres

souches de *B. cereus* *sl.* Une dizaine de HK et RR sont « orphelins », c'est-à-dire qu'ils ne sont putativement pas appariés chacun à un partenaire RR ou HK.

Tableau 6 : Nombre de HK et RR présent dans le génome de différentes souches de *B. cereus* *sl.* et une souche de *B. subtilis* (Mark de Been, 2006 (de Been et al., 2006a))

| Species | HKs | RRs | HK-RR gene pairs | HK-RR fusions | 'Orphans' | |
|--|-----|-----|---------------------|------------------|-----------|-----|
| | | | | | HKs | RRs |
| <i>B. cereus</i> ATCC 14579 | 55 | 48 | 39 | 2 | 14 | 7 |
| <i>B. cereus</i> ATCC 10987 | 54 | 49 | 40 | 2 | 12 | 7 |
| <i>B. cereus</i> ZK | 57 | 52 | 43 | 2 | 12 | 7 |
| <i>B. thuringiensis</i> <i>konkukian</i> | 58 | 52 | 44 | 1 | 13 | 7 |
| <i>B. anthracis</i> Ames (0581), Sterne | 52 | 51 | 41 | 1 | 10 | 9 |
| <i>B. anthracis</i> A2012 | 50 | 50 | 38 | 1 | 11 | 11 |
| <i>B. subtilis</i> 168 | 35 | 35 | 29 | 0 | 6 | 6 |

La Figure 16 présente une vue globale de ces TCS chez *Bacillus cereus sensu lato* (Abee et al., 2011a) ainsi que le rôle biologique putatif de chaque TCS. La majorité des TCS, environ 40 TCS, sont appariés et y sont représentés. Des connexions entre les HK et RR orphelins ont été prédits *in silico* et sont représentés par des flèches grises sur la Figure 16. Par exemple plusieurs HK orphelines ont été associés aux protéines d'initiation de phosphorylation Spo0F, spo0A et spo0B.

Quelques HK cytoplasmiques se distinguent de la grande majorité de HK membranaires. C'est le cas des HK 39, 43, 44, 46, 47, 63 mais aussi YhcY (HK62).

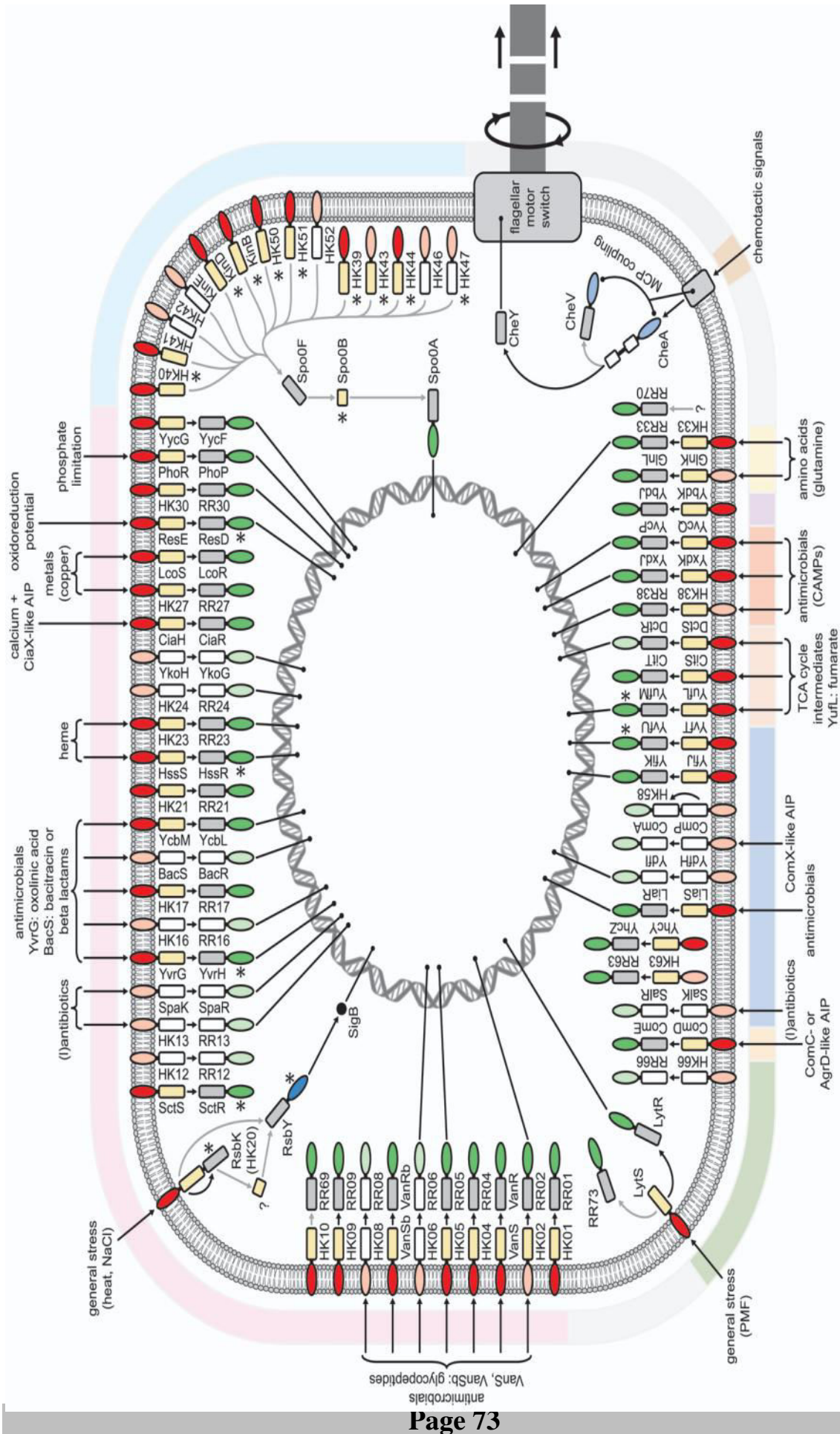


Figure 16 : Vue globale des TCS présents chez des souches de *B. cereus* *sl* ainsi que leur rôle putatif. (Mark de Been, 2011 (Abee et

IV) Rôle des TCS dans l'adaptation au froid

Les TCS impliqués dans l'adaptation à divers changements environnementaux, peuvent parfois percevoir et favoriser la réponse adaptative à une baisse de température. La température est un facteur environnemental qui d'une part est utilisé lors de la conservation et la transformation des aliments, mais aussi qui peut conditionner l'adaptation d'espèces alimentaires pathogènes telles que celles du groupe *B. cereus* s1 (Guinebretiere et al., 2008b).

La conservation aux basses températures est une étape critique dans la sécurité des aliments, d'autant plus qu'en général cette étape précède la consommation des aliments. Pourtant, comme précédemment énoncé, certaines souches pathogènes de *B. cereus* s1 sont capables de se développer dans l'aliment réfrigéré.

De manière générale, de nombreux mécanismes sont mis en place par les bactéries pour répondre à une baisse de température environnementale (Figure 17).

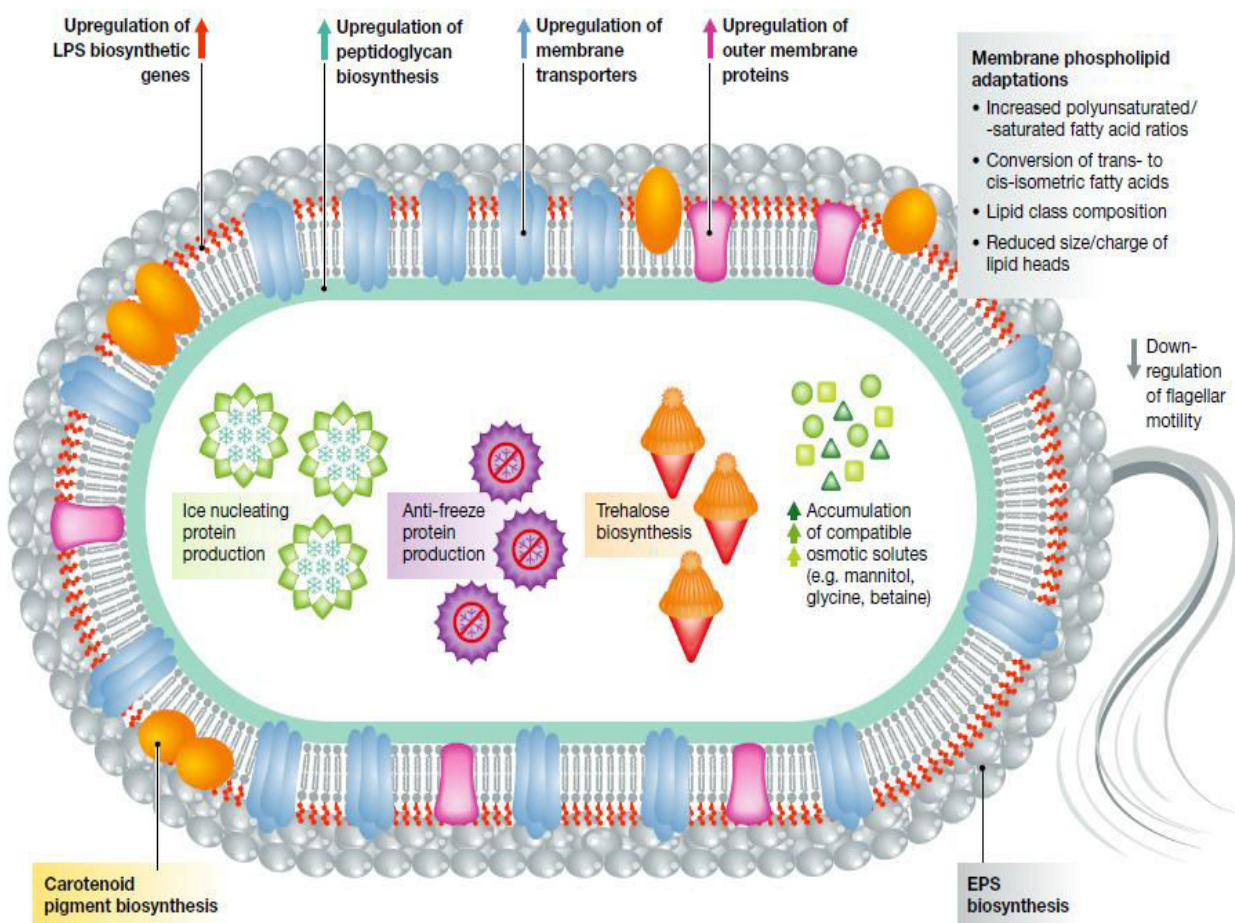


Figure 17 : mécanismes d'adaptations physiologiques au froid rencontrés chez les procaryotes. (LPS=lipo-polysaccharides)(De Maayer et al., 2014)

L'adaptation au froid chez les souches du groupe *B. cereus*, comme chez d'autres bactéries, est aussi un mécanisme multifactoriel (Requena, 2012). En effet plusieurs études menées à ce propos ont permis d'identifier des facteurs tels que :

-les hélicases à ARNs sont des protéines chaperonnes des ARNs leur permettant de maintenir une bonne conformation pour que les mécanismes de traductions soient maintenus à basse température (Broussolle et al., 2010a, Pandiani et al., 2010a, Pandiani et al., 2011a),

-les « cold shock protein » sont des protéines induites immédiatement après une chute de température (Graumann and Marahiel, 1996a), contenant un domaine « cold shock » très conservés et jouant un rôle dans le maintien de la transcription et la traduction lors d'un choc froid.

-la membrane dont le maintien de la fluidité à basse température est cruciale à l'adaptation au froid (de Sarrau et al., 2012b). Le maintien de cette fluidité chez *Bacillus cereus* au froid se fait par une augmentation des acides gras insaturés de la membrane (de Sarrau et al., 2012b).

-une lipase, enzyme lipolytique dont le rôle n'est pas clairement défini, serait aussi impliqué dans l'adaptation au froid (Brillard et al., 2010b)

Aucune donnée concernant l'implication un système à deux composants dans cette adaptation n'a été décrit dans la littérature scientifique.

Il existe assez peu d'études focalisées sur l'implication des systèmes à deux composants dans l'adaptation au froid des bactéries (Tableau 7). Certaines ont été réalisées chez les bactéries à Gram négatif telles que *Caulobacter crescentus* (Wu et al., 1998), *Flavobacterium psychrophilum* (Hesami et al., 2011) et *Synechocystis* sp. PCC 6803 (Mironov et al., 2012). D'autres études existent chez les bactéries à Gram positif telles que *Prochlorococcus* MED4 (Mary and Vaultot, 2003), *Clostridium botulinum* (Derman et al., 2013) et une espèce du même genre que les souches de *B. cereus* *sl*, *B. subtilis* (Aguilar et al., 2001b)

Dans la plupart des cas, les études s'arrêtent à des descriptions phénotypiques de ces TCS dans l'adaptation, le mécanisme par lequel cela est possible est très peu décrit.

A titre d'exemple, CBO2306/CBO2307 et CBO366/CBO365 ont été identifiés chez *Clostridium botulinum* ATCC 3502 et les mutants de ces gènes impactaient la capacité de cette bactérie à se développer au froid (Derman et al., 2013, Lindström et al., 2012). Ces TCS ont été décrit comme impliqués dans l'adaptation au choc froid chez *C. botulinum*.

L'histidine kinase Hik33 de *Synechocystis* sp. PCC 6803 est un peu mieux décrite, cette HK régule un ensemble de gènes nécessaires pour l'adaptation au froid. Cette régulation dépend d'une part de la fluidité membranaire dont la modification semble être reconnue par Hik33, mais aussi de la lumière, *Synechocystis* sp. PCC 6803 étant une bactérie photo-synthétique (Mironov et al., 2012).

Tableau 7 : liste des TCS identifiés dans la littérature comme impliqués dans l'adaptation au froid chez différentes bactéries.

| ESPECES | TCS | ROLE | REFERENCES |
|-------------------------------------|----------------------------------|---|---|
| <i>Caulobacter crescentus</i> | DivJ/DivK PleC et PleD | Impact sur la croissance et la mobilité et la division au froid | (Burton et al., 1997, Wu et al., 1998) |
| | SpdR | Régulation de « cold shock » protéines | (da Silva et al., 2010) |
| <i>Flavobacterium psychrophilum</i> | LytS | Surexprimés à basse température | (Hesami et al., 2011) |
| <i>Synechocystis</i> sp. PCC 6803 | Hik33 | Impliqué dans la régulation de gènes et de la fluidité membranaire pour l'adaptation au froid | (Mironov et al., 2012) |
| <i>Prochlorococcus</i> MED4 | Six HKs et cinq RRs | Surexprimés à basse température | (Mary and Vaultot, 2003) |
| <i>Clostridium botulinum</i> | CBO2306/CBO2307 CBO366/CBO365 | Impact sur la croissance au froid | (Derman et al., 2013, Lindström et al., 2012) |
| | CLO3403/3404 | Impact sur la croissance et la mobilité au froid | (Mascher et al., 2014) |
| <i>B. subtilis</i> | DesK/DesR | Impliqué dans la régulation de gènes et de la fluidité membranaire pour l'adaptation au froid | (Aguilar et al., 2001b) |

Le système le mieux décrit pour l'adaptation au froid est le système DesK/DesR de *Bacillus subtilis*. DesK est une HK membranaire qui lors d'un stress froid va être capable de sentir la

perte de la fluidité membranaire de la bactérie (Figure 18), la rigidification de la membrane entraînant un changement conformationnel de cette protéine (Inda et al., 2014b). Puis cette protéine va s'autophosphoryler (Trajtenberg et al., 2010, Martin and de Mendoza, 2013) et transmettre le groupement phosphate à DesR (Cybulski et al., 2004, Albanesi et al., 2004). DesR va réguler l'expression du gène *des* codant pour l'unique désaturase de *B. subtilis* (Aguilar et al., 2001b) est responsable de la formation des insaturations des acides gras (Aguilar et al., 2001b). Activée à basse température, elle est responsable de l'augmentation des acides gras insaturés dans la membrane et donc de l'homéostasie de la fluidité membranaire dans cette même condition.

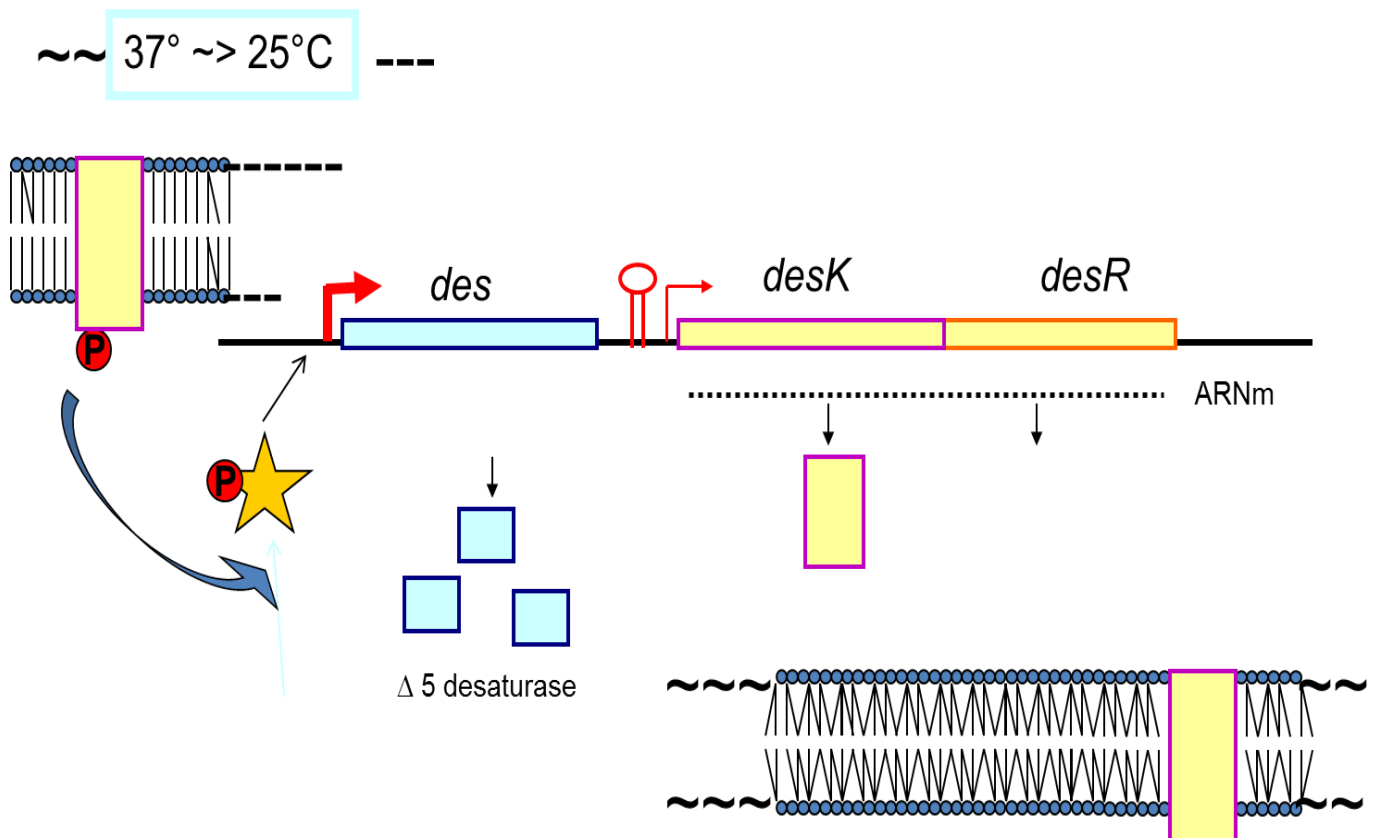


Figure 18 : Mécanisme d'adaptation de *B. subtilis* au froid médié par DesK/DesR

V) Présentation du sujet de thèse

Bacillus cereus sensu lato est un groupe de bactéries incluant des pathogènes connus tels que *B. cereus*, *B. anthracis* et *B. thuringensis* et faisant preuve de grandes capacités adaptatives. Parmi ceux-ci on retrouve des pathogènes alimentaires tels que *B. cereus ss* et *B. cytotoxicus*. L'adaptation et la capacité de croissance aux basses températures du pathogène alimentaire *B. cereus* représente un aspect critique par rapport à la conservation des aliments au froid.

La maîtrise du risque associé à *B. cereus* dans les aliments réfrigérés sous-entend de mieux appréhender les mécanismes moléculaires clés impliqués dans l'adaptation de cette bactérie au froid. Cela permettrait de mieux prédire le comportement de ces bactéries durant la latence et donc l'acclimatation aux basses températures rencontrées lorsque les aliments sont réfrigérés, chose peu décrite avec les modèles mathématiques de prédiction de croissance existants.

B. cereus représente un modèle intéressant car cette espèce contient des souches qui présentent des thermotypes variés. L'étude de l'adaptation au froid sur la base de cette diversité de thermotypes représente une approche intéressante pour identifier un mécanisme commun d'adaptation des souches de *B. cereus sl* aux basses températures.

Les TCS représentent un des principaux mécanismes d'adaptation des bactéries aux environnements, ainsi un criblage des gènes codant pour ces systèmes et potentiellement impliqués dans l'adaptation au froid de *B. cereus* a été réalisée dans notre laboratoire. Un TCS dont les gènes sont surexprimés à basse température a été sélectionné. L'étude *in silico* de ce TCS a permis de mettre en évidence qu'il est conservé chez l'ensemble des souches des groupes phylogénétiques de *B. cereus sl* excepté dans deux groupes, dont celui regroupant les souches les plus thermotolérantes. Ce TCS représentait par conséquent un candidat intéressant pour notre étude. L'ensemble des travaux de recherche que je présente dans ce manuscrit s'est donc focalisé sur la compréhension des mécanismes de fonctionnement de ce TCS et la mise en évidence de son rôle générique au sein du groupe *B. cereus*.

Ainsi, l'étude de ce TCS ainsi que la caractérisation de ces mutants isogéniques obtenus chez différentes souches du groupe *B. cereus sl* seront détaillées dans le **chapitre 2**. Puis, la

description des mécanismes moléculaires impliquant ce TCS et mis en évidence durant mes travaux de recherches sera abordée dans les **chapitres 3 et 4** de ce manuscrit. Des informations complétant ces travaux de recherches seront développés dans le **chapitre 5**.

Chapitre 2 :

CasK/R est un système générique
impliqué dans l'adaptation aux basses
températures de *Bacillus cereus sensu*
lato

Chapitre 2: CasK/R est un système générique impliqué dans l'adaptation aux basses températures de *Bacillus cereus sensu lato*

I- Introduction à l'étude

B*acillus cereus sensu lato (sl)* est constitué de 7 espèces génétiquement très proches, incluant des pathogènes connus comme *B. anthracis*, *B. thuringensis* et *B. cereus sensu stricto (ss)*. Les souches de *B. cereus sl* sont réparties en 7 groupes phylogéniques caractérisés par différentes gammes de températures de croissances que l'on peut globalement répartir en 4 thermotypes : des souches psychrotolérantes (groupes 6 et 2), intermédiaires mésophile-psychrotolérant (groupe 5) mésophiles (groupes 1, 3, 4) et thermophiles (groupe7) (Guinebretière et al., 2008, Guinebretière et al., 2013). Les souches pathogènes isolées de toxi-infections alimentaires collectives (TIAC) sont généralement les espèces *B. cereus ss* et *B. cytotoxicus* causant des syndromes émétiques et/ou diarrhéiques, (Guinebretiere et al., 2013b, Stenfors Arnesen et al., 2008c, Guinebretiere et al., 2010). Toutefois, certaines souches de *B. weihenstephanensis*, une espèce psychrotolérante de *B. cereus sl*, peuvent parfois produire le céréulide, la toxine causant le syndrome émétique (Rossvoll et al., 2014)

La dose minimale infectante (DMI) de *B.cereus ss* de 10^5 UFC/ml implique une croissance des souches dans l'aliment avant consommation. Les souches psychrotolérantes représentent donc un danger alimentaire étant donné qu'elles sont capables de se développer à des températures telles que celles rencontrées dans la chaîne du froid pour la conservation des aliments. Comprendre les mécanismes impliqués dans cette adaptation permettrait à terme d'enrichir les modèles de prédictions préexistants sur le développement de bactéries pathogènes, avec notamment plus de données sur la phase d'acclimatation ou de latence à basse température.

L'adaptation cellulaire au froid est connue pour être multifactorielle. Plusieurs mécanismes semblent impliqués dans l'adaptation au froid chez *B. cereus* : les « cold shock » protéines (Csps) (Mayr et al., 1996), les hélicases à ARNs (Pandiani et al., 2010a, Pandiani et al., 2011a), une lipase (Brillard et al., 2010c), les composants de la membrane (de Sarrau et al., 2012c, de Sarrau et al., 2013d, Haque and Russell, 2004b).

Très peu d'études décrivent à ce jour l'implication d'un système à deux composants (TCS) dans l'adaptation au froid, en particulier dans le genre *Bacillus*. Un TCS est néanmoins très bien décrit à ce propos, il s'agit de DesK/R de *B. subtilis* (Aguilar et al., 2001b).

Cependant, jusqu'à lors, aucune étude n'a mis en évidence l'implication de systèmes à deux composants (TCS) dans l'adaptation de *B. cereus* au froid bien que ces systèmes soient des mécanismes majeurs d'adaptation des bactéries à des environnements stressants.

Existe-t-il un ou plusieurs TCS impliqué(s) dans l'adaptation au froid chez *B. cereus* ? Pour répondre à cette question, l'expression de plusieurs TCS a été mesurée à basse température et comparée aux valeurs obtenues à température optimale de croissance lors d'une étude du transcriptome de la souche type de *B. cereus* ATCC 14579.

Ces résultats ont permis d'identifier un système à deux composants (YhcY/Z), surexprimé à basse température et très conservés chez *B. cereus sl*. Un paralogue de ce TCS identifié dans le génome de la souche type mais celui-ci n'était pas surexprimé à basse température.

Nous avons donc tenté de répondre à plusieurs questions :

Le système à 2 composants YhcY/Z joue -t-il un rôle dans l'adaptation de *Bacillus cereus* au froid ? En est-il de même pour son paralogue ?

Qu'en est-il pour les souches de *B. cereus sl* présentant un thermotype différent de la souche ATCC 14579 et chez lesquelles ce système à deux composants est conservé ?

II- Stratégies envisagées

Un mutant isogénique de *yhcY/Z* a été construit par échange allélique avec une cassette conférant la résistance à la kanamycine (Km^r), (s'exprimant en anti-sens par rapport aux gènes pour éviter tout effet polaire) comme précédemment décrit (Arnaud et al., 2004a). Ce mutant a été caractérisé phénotypiquement après avoir été soumis à différentes conditions stressantes parmi lesquelles la croissance et la survie au froid. Après comparaison des comportements des souches WT et mutante, les phénotypes dus à la mutation ont été validés par complémentation en *trans* de la souche mutante. En parallèle à ces expériences, un mutant

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du paralogue de *yhcY/Z* a été construit chez la même souche et étudié phénotypiquement, mais aucun phénotype froid n'a été observé.

Après cela, des mutants isogéniques de ce TCS ont été construits chez des souches ayant des thermotypes différents de celui de la souche modèle testée, le but étant de définir si le comportement de ces mutants au froid était similaire à celui du mutant obtenu chez la souche type. Nous avons donc sélectionné trois autres souches appartenant à d'autres groupes phylogénétiques et ayant un génome séquencé disponible. Ce sont: La souche AH187 (groupe III, souche plus mésophile que l'ATCC 14579), MM3 (group II, souche psychrotolérante) et Rock 3-28 (group V, souche intermédiaire mésophile-psychrotolérante). Leur comportement à basse température a été étudié.

III- Résultats et discussion

La caractérisation de la souche $\Delta yhcY/Z$ chez la souche ATCC 14579 a permis de mettre en évidence que ce mutant était affecté dans sa croissance à basse température (mais pas dans le cas des autres stress testés). Ceci se manifeste par une absence de croissance à T_{min} (10°C) et un retard de croissance à 12°C, comparé à la souche WT. La survie de la souche mutante à 4°C s'est aussi révélée beaucoup moins bonne que celle de la souche WT. La structure des cellules de la souche mutée était aussi altérée avec une paroi large et d'épaisseur irrégulière, contrairement à la souche WT présentant une paroi fine et d'épaisseur régulière. Ainsi, au vu de l'implication de ce TCS dans l'adaptation de *B. cereus* au froid, celui-ci a été renommé CasK/R, Cas pour « cold adaptation system ». Il est constitué de l'histidine kinase CasK et du régulateur de réponse CasR.

Suite à la complémentation en *trans* de la souche mutante $\Delta casK/R$ avec une copie de *casK/R*, cette souche présente des phénotypes identiques à la souche WT à basse température. Cela montre bien que CasK/R est impliqué dans l'adaptation au froid de *B.cereus*.

Comme indiqué précédemment le mutant du paralogue de *casK/R* a aussi fait l'objet de notre étude. Celui-ci ne présente aucun phénotype froid suggérant que ce paralogue n'est pas impliqué dans l'adaptation au froid.

Les mutants de *casK/R* ont été construits chez des souches de thermotypes différents de la souche type et quel que soit la souche de *B. cereus sl* testée, le mutant $\Delta casK/R$ est affecté

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dans sa croissance au froid en comparaison de la souche parentale correspondante. Cette observation est vérifiée indépendamment du thermotype.

Il est à noter que pour cette étude nous avons aussi sélectionné des souches appartenant au groupe le plus psychrotolérant, le groupe 6 de *B. cereus sl.* Cependant, muter les souches de ce groupe s'est avéré impossible et ce très probablement à cause de la limite de la méthode expérimentale utilisée. En effet, le vecteur plasmidique utilisé pour nos expériences, le plasmide pMAD (Arnaud et al., 2004b), possède une origine de réplication thermosensible, inductible à 42°C, température supérieure à la température maximale de croissance des souches psychrotolérantes. Même si pour les souches des groupes 5 et 2 utilisées, en forçant l'induction respectivement à 40°C ou 37°C, il a été possible d'obtenir des mutants, pour les souches du groupe 6 ayant une limite de croissance de 37°C, cela a été impossible, même en réalisant les inductions à cette température, d'obtenir ne serait-ce que des simples recombinants. Les essais de mutation dans cette souche ont été abandonnés car un mutant de *casK/R* avait malgré tout été obtenu chez une autre souche psychrotolérante (la souche MM3). Les résultats obtenus chez la diversité de souches de *B. cereus sl* testées a permis de démontrer clairement le rôle générique de CasK/R dans l'adaptation au froid des souches de ce groupe.

The CasKR two-component system is required for growth at low temperature of mesophilic and psychrotolerant *Bacillus cereus* strains

Sara Diomandé^{1,2}, Stéphanie Chamot^{1,2}, Vera Antolinos^{3,4}, Florian Vasai^{1,2}, Marie-Hélène Guinebretière^{1,2}, Isabelle Bornard⁵, Christophe Nguyen-the^{1,2}, Véronique Broussolle^{1,2} and Julien Brillard^{1,2}#.

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¹ INRA, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France

² Université d'Avignon et des Pays de Vaucluse, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France.

³ Instituto de Biotecnología Vegetal, Universidad Politécnica de Cartagena, Cartagena, Spain

⁴ Dpto. Ingeniería de Alimentos y del Equipamiento Agrícola, Escuela Técnica Superior de Ingeniería Agronómica, Universidad Politécnica de Cartagena, Cartagena, Spain

⁵ INRA, UR407 Pathologie Végétale, F- 84140 Montfavet, France

Corresponding author: julien.brillard@avignon.inra.fr

Running title: CasKR requirement for *B. cereus* cold growth

Les références de chaque article sont toutes classées dans la partie Références du manuscrit (page 188)

Abstract

The different strains of *Bacillus cereus* can grow in a very diverse range of temperatures. Some *B. cereus* strains can grow in chilled food and consequently cause food poisoning. We have identified a new sensor/regulator mechanism involved in low-temperature *B. cereus* growth. Construction of a mutant of this two-component system enabled us to show that this system, called CasKR, was required for growth at the minimal temperature (T_{min}). CasKR was also involved in optimal cold growth above T_{min} and in cell survival below T_{min}. Microscopic observation showed that CasKR plays a key role in cell shape during cold growth. Introducing the *casKR* genes in the Δ *CasKR* mutant restored its ability to grow at T_{min}. Although first identified in the ATCC 14579 model strain, this mechanism has been conserved in most strains of the *B. cereus* group. We showed that the role of CasKR in cold growth was similar in other *B. cereus* sl strains with different growth temperature ranges, including psychrotolerant strains.

Keywords:

B. cereus, 2CS, YhcYZ, cold, survival, psychrotroph

Introduction

The *Bacillus cereus* group, also known as *B. cereus sensu lato* (*B. cereus sl*) consists of ubiquitous Gram-positive spore-forming bacteria. This group comprises seven closely-related species, including *B. cereus sensu stricto* (*B. cereus ss*) and *B. cytotoxicus* that cause emetic and/or diarrheal syndromes in food-poisoning (Guinebretiere et al., 2013b, Stenfors Arnesen et al., 2008c, Guinebretiere et al., 2010). Some rare strains of *B. weihenstephanensis*, another species of this bacterial group, can also be emetic (Thorsen et al., 2006a). These bacteria cause a number of food safety issues, as they are able to produce heat-resistant spores and thus survive in food even after processes such as cooking or pasteurization. Compared to *B. cereus ss*, food poisoning by *B. cytotoxicus* or *B. weihenstephanensis* is rare (Guinebretiere et al., 2013b, Guinebretiere et al., 2010). The risk for consumers is mostly tied to high doses (10^5 – 10^8 CFU) of cells or spores contaminating the ingested food, and thus depends on the ability of the bacterium to multiply during the food's shelf-life (Brillard and Broussolle, 2012, Carlin et al., 2010, Stenfors Arnesen et al., 2008c). As some strains of *B. cereus* have the ability to grow at temperatures found in the chill chain, identifying the mechanisms involved in low-temperature adaptation may help predict how this bacterium behaves in refrigerated food and enable more accurate risk prediction and better risk prevention.

Bacterial adaptation to low temperature is a complex and multifactorial process involving both the genetic background of the bacteria (Guinebretiere et al., 2008a) and an array of mechanisms (Brillard and Broussolle, 2012). Concerning the genetic background, seven phylogenetic groups (I-VII) were defined in *B. cereus sl* in which *B. cereus ss* was broadly positioned (groups II to VI), while *B. weihenstephanensis* and *B. cytotoxicus* were merged with group VI and VII, respectively (Guinebretiere et al., 2008a). *B. cereus ss* is thus expected to show a broadly-diverse genetic background, in contrast to *B. cytotoxicus* or *B. weihenstephanensis*. Interestingly, each phylogenetic group (I-VII) was assigned a specific range of growth temperatures based on both genetic and phenotypic criteria (Guinebretiere et al., 2008a). These seven phylogenetic groups could also be seen as seven 'thermotypes', running from psychrotolerant (cold-tolerant) to moderate psychrotolerant, mesophile and moderate thermotolerant (heat-tolerant) groups. *B. cereus ss* thus spanned different thermotypes containing psychrotolerant, mesophilic or intermediate strains, whereas *B. weihenstephanensis* contained only psychrotolerant strains and *B. cytotoxicus* contained only moderate thermotolerant strains. It was suggested that in the course of evolution, changes in

temperature tolerance limits have fashioned historical patterns of global ecological diversification in *B. cereus sl* (Guinebretiere et al., 2008a).

The mechanisms involved in adaptation to low temperature are equally multifactorial, as illustrated by the various mechanisms identified so far in other model bacteria and also described in *B. cereus* (Brillard and Broussolle, 2012). For instance, membrane fatty acid (FA) composition adjustments can increase the proportion of low-melting-point FA (like unsaturated FA, branched-chain FA) (Brillard et al., 2010a, de Sarrau et al., 2012a, Haque and Russell, 2004a). RNA helicases that enable the RNA unfolding needed for proper translation and/or RNA degradation also play a major role in *B. cereus* low-temperature adaptation (Pandiani et al., 2010b).

Among the mechanisms that allow organisms to changes in response to environmental conditions, two-component systems (2CS) are signal transduction systems that are almost ubiquitous in bacteria (Galperin, 2005a). 2CS are known to sense a wide range of environmental stressors, enabling cells to elaborate a response by regulating the expression of genes required for adaptation (Galperin, 2010, Krell et al., 2010, Mascher et al., 2006a, Szurmant et al., 2007, Whitworth and Cock, 2009). 2CS basically comprise a histidine kinase (HK) that senses an environmental stimulus (either directly or after interaction with accessory proteins) and a cognate response regulator (RR) that usually functions as a transcriptional regulator. One of the first studies on a 2CS involved in cold adaptation identified DesKR, which is able to sense an increase in membrane thickness in response to a decrease in temperature in *B. subtilis* (Aguilar et al., 2001c, Cybulski et al., 2010a). DesKR consequently regulates the expression of a desaturase gene responsible for maintaining membrane fluidity during *B. subtilis* growth at low temperature. More recently, a *Clostridium botulinum* 2CS important for cold tolerance was discovered, and the mechanisms involved were investigated (Dahlsten et al., 2014b, Lindstrom et al., 2012). In a Gram-negative bacterium, the CheA/CheY 2CS mutant of *Yersinia pseudotuberculosis* was impaired during growth at 3°C (Palonen et al., 2011). 2CS of other Gram-negative bacteria have been described as temperature sensors for bacterial virulence control, such as CorSR in *Pseudomonas* (Braun et al., 2008) and PhoPQ in *Edwardsiella* (Chakraborty et al., 2010). Recent research has revealed how 2CS contribute to the high adaptability of *B. cereus* strains that enable these bacteria to persist in processed foods (Abee et al., 2011b). Some 2CS were shown to play diverse roles in *B. cereus* adaptation (Brillard et al., 2008a, de Been et al., 2010, Duport et al., 2006a, Fagerlund et al., 2007, Song et al., 2012a), but most of the 2CS found among *B. cereus*

sl strains have unknown function (de Been et al., 2006b) and none has yet been shown to be involved in low-temperature adaptation.

In the course of experiments to better understand the role of the numerous 2CS with unknown function found among *B. cereus sl* strains, we mutated one of them (BC_2216-BC_2217) in the mesophilic model *B. cereus ss* strain ATCC 14579. We found that this new 2CS was necessary for low temperature adaptation not only in mesophilic strains but also in two psychrotolerant strains belonging to distinct phylogenetic groups of *B. cereus sl*.

Materials & Methods

Strains and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *B. cereus* cells were grown in Luria broth (LB) or brain heart infusion (BHI) with vigorous shaking (200 rpm) at 37°C, 30°C, 13°C, 12°C, 8°C or 10°C. *E. coli* cells were routinely grown in LB medium with shaking at 37°C. When required, the antibiotic concentrations used for bacterial selection were: erythromycin (Em) at 10 µg ml⁻¹, kanamycin (Km) at 150 µg ml⁻¹ or spectinomycin (Sp) at 275 µg ml⁻¹ for *B. cereus* and ampicillin at 100 µg ml⁻¹ for *E. coli*. *E. coli* ET12567 *dam*⁻ *dcm*⁻ was used to generate unmethylated plasmid DNA for the electrotransformation of *B. cereus*. *B. cereus* and *E. coli* strains were transformed by electroporation as previously described (Dower et al., 1988, Lereclus et al., 1989). When appropriate, the lag phase (defined as initial OD + 0.05), time to reach stationary phase, or maximal OD were indicated to compare bacterial growth curves during various conditions.

To compare the growth of phylogenetic group II, III and V mutants against their parental strains, an automated turbidimeter (Microbiology Bioscreen C Reader, Labsystems, Uxbridge, UK) was used as previously described (Brillard et al., 2010a).

Cultures were performed as follows. A fresh colony was used to inoculate 10 ml LB and incubated at 30°C with shaking for 9 hours. Ten µl were then used to inoculate 10 ml fresh LB, and incubated at 30°C with shaking for 17 hours. This second culture was used to inoculate 1 ml fresh LB to reach a concentration of 10⁵ CFU per ml. Three replicate microplate wells were filled with dilutions of inoculated medium to a final volume of 300 µl per well.

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A negative control was made of un-inoculated LB broth. Cultures were incubated under vigorous constant shaking, and OD₆₀₀ was measured at 15-min intervals at 37°C, 42°C, 45°C or at 1-h intervals at 13°C, 12°C, 10°C or 8°C over an incubation period of 48 h (at 37°C and above) or 10 days (for all other temperatures). At least three independent experiments were performed for each growth condition.

Table 1. Strains and plasmids used in this work.

| Strain or plasmid | Relevant genotype and characteristics ^a | Reference or source |
|----------------------------------|--|---|
| Strains | | |
| <i>B. cereus</i> ATCC 14579 WT | ATCC 14579, wild type, phylogenetic group IV | ATCC, USA |
| ATCC 14579 $\Delta casKR$ | WT ^{$\Delta BC_{2216-BC_{2217}}$} , Km ^r | This study |
| ATCC 14579 $\Delta BC_{5411-12}$ | WT ^{$\Delta BC_{5412-BC_{5411}}$} , Sp ^r | This study |
| <i>B. cereus</i> AH187 WT | AH187=F4810/72, wild type, phylogenetic group III | PHLS, UK |
| AH187 $\Delta casKR$ | WT ^{$\Delta BCAH187_{A2374-A2375}$} , Km ^r | This study |
| <i>B. cereus</i> MM3 WT | MM3, wild type, phylogenetic group II | Naval Medical Research Center (US NAVY) |
| MM3 $\Delta casKR$ | WT ^{$\Delta bcere0006_{20650-20660}$} , Km ^r | This study |
| <i>B. cereus</i> Rock 3-28 WT | Rock 3-28, wild type, phylogenetic group V | Naval Medical Research Center (US NAVY) |
| Rock 3-28 $\Delta casKR$ | WT ^{$\Delta bcere0019_{20130-20120}$} , Km ^r | This study |
| <i>E. coli</i> TG1 | $\Delta(lac-proAB)$ <i>supE thi hsd-5</i> (<i>F'</i> <i>traD36 proA</i> ⁺ <i>proB</i> ⁺ <i>lacI</i> ^q <i>lacZ</i> Δ M15), general purpose cloning host | Laboratory collection |
| <i>E. coli</i> SCS110 | <i>rpsL</i> (<i>Str</i> ^r) <i>thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44 $\Delta(lac-proAB)$ [<i>F'</i> <i>traD36 proAB lacI</i>_qΔM15], for generation of unmethylated DNA</i> | Laboratory collection |
| plasmids | | |
| pHT304-18 | Ap ^R and Em ^R cloning vehicle | (Arantes and Lereclus, 1991) |
| pHT304-Km | pHT304-18 derivative harbouring an Km ^r cassette | (Brillard et al., 2008a) |
| pMAD | Ap ^r , Em ^r shuttle vector, thermosensitive origin of replication | (Arnaud et al., 2004b) |
| pMAD $\Delta casKR$ | pMAD derivatives harbouring constructs for the allelic replacement of <i>casK-casR</i> , Ap ^r , Em ^r , Km ^r | This study |
| pHT- <i>casKR</i> | 2848 bp region surrounding <i>casK</i> and <i>casR</i> genes cloned in <i>SalI</i> and <i>BamHI</i> sites of pHT304-18 | This study |
| pMAD $\Delta BC_{5412-11}$ | pMAD derivatives harbouring constructs for the allelic replacement of BC _{5412-BC₅₄₁₁} , Ap ^r , Em ^r , Sp ^r | This study |

^a Km, kanamycin; Ap, ampicillin; Em, erythromycin, Sp, spectinomycin

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The same protocol was used for low-pH (5.5, 5.0 and 4.7), high-pH (8.5 and 8.6) and high-osmolarity conditions (4 and 6 % NaCl) in presence of oxidative agents (Paraquat dichloride 100 μ M and 150 μ M; AAPH (2-2' azo-bis-[2-methyl-propionamidine] dihydrochloride) 100 mM and 150 mM; H₂O₂ 0.020% and 0.025%). Alternatively, growth was performed in 50 ml LB placed in 250 ml Erlenmeyer flasks in presence of ethanol (4% and 6%), ions chelators (EDTA 100 μ M; EGTA 100 μ M; 2,2'-bipyridyl 100 μ M and 500 μ M), and OD₆₀₀ was regularly checked on a Helios Epsilon spectrophotometer (Thermo Scientific, Rockford, IL). All chemicals were purchased from Sigma-Aldrich.

Standard tests for *B. cereus*, such as growth on Mossel (also called MYP for Mannitol Egg Yolk Polymyxin) Agar or on sheep blood Agar were performed as described in Bergey's Manual (Claus and Berkeley, 1986), and API50CH strips (BioMérieux, Marcy-L'Etoile, France) were used as per the manufacturer's protocol.

Microscopic observations

Bacterial cells were regularly observed under phase-contrast microscopy at a magnification of x1000. To visualize cell shape at a higher magnification (up to x14000), transmission electron microscopy was used as previously described (de Sarrau et al., 2013b, Pandiani et al., 2010b). Briefly, wt and mutant cells grown at 12°C or 37°C were harvested in early stationary phase and transferred to glutaraldehyde at 2.5% in 0.1 M sodium cacodylate buffer (pH 7.2) containing ruthenium red (1 mg/ml). Cells were washed three times and post-fixed with 2% osmium tetroxide, washed again, mixed with ethanol 30%, then embedded in 3% agar before dehydration with increasing concentrations of ethanol. Ethanol was replaced with propylene oxide and sequentially exchanged with araldite resin. Samples were polymerized for 48 h at 60°C before cutting. Thin 70 nm-wide sections were cut with an ultramicrotome and stained with uranyl acetate and lead citrate. Observations were done under a Transmission Electron Microscope (TEM e FEI-Philips CM10, Eindhoven, The Netherlands).

Cell survival experiments

Cell survival at 4°C was tested by inoculating 2.5 mL LB in 10 ml tubes with exponential-phase subcultures of wt and mutant cells and incubating at 4°C for up to 840 hours (35 days). The number of survivors was determined by plating 100- μ L volumes of 10-fold serial dilutions of the culture stored on triplicate LB agar plates, and counting the colonies formed after 18 h of incubation at 30°C. This experiment was performed in triplicate. Strain survival

at 4°C was modeled as previously described using the function $\log N = \log N_0 - (t / \delta) p$, where N_0 is initial population, N is population at time t , δ is time to first decimal reduction, and p is curvature index (Mafart et al., 2002). To model the shape of the curve, δ and p were calculated using the Microsoft Excel 2010 solver function.

Nucleic acid manipulations

Plasmid DNA was extracted from *B. cereus* and *E. coli* by a standard alkaline lysis procedure using the Wizard SV miniprep purification system (Promega, Charbonnières, France), with an additional incubation with lysozyme to lyse *B. cereus* cells as previously described (Brillard and Lereclus, 2007a). Chromosomal DNA was extracted from *B. cereus* cells harvested in mid-log phase as described previously (Brillard and Lereclus, 2007a). Restriction enzymes and T4 DNA ligase were used as recommended by the manufacturer (Promega). Oligonucleotide primers (Table 2) were synthesized by Eurogentec (Seraing, Belgium). PCR was performed in a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer, Courtaboeuf, France) using Expand high-fidelity DNA polymerase (Roche Applied Science, Meylan, France). Amplified DNA fragments were purified using a PCR purification Kit (Roche) and separated on 0.7% agarose gels after digestion as previously described (Brillard and Lereclus, 2007a). Digested DNA fragments were extracted from agarose gels with a centrifugal filter device (Montage DNA gel extraction kit, Millipore, Molsheim, France). All constructions were confirmed by DNA sequencing (Millegen, Labège, France).

RNA extraction and real-time RT-PCR were performed on a LightCycler instrument (Roche) using a QuantiFast SYBR green RT-PCR kit (Qiagen) as previously described (Brillard et al., 2010a), with the primer pair qPCR-BC2216-F and qPCR-BC2216-R (Table 2).

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Table 2. Primers used in this study.

| oligonucleotides | sequence (5' - 3') ^a |
|--------------------------|---|
| <i>5Up-2216-17-Eco</i> | GGCG <u>AATTC</u> GTAGTTGGTGATGAGAATGAAC |
| <i>3Up-2216-17-Pst</i> | TGGAC <u>TGCAGCA</u> ATCCTTCAGGTCTTAATTG |
| <i>5Dn-2216-17-Sal</i> | TGCAG <u>TGCAGC</u> GAAAACGAGAGAAGAATTTGA |
| <i>3Dn-2216-17-Bam</i> | GCGGG <u>ATCCTTT</u> ATCCTGTAAAAACCGATTA |
| <i>Km5out</i> | CGGTATAATCTTACCTATCACC |
| <i>Km3out</i> | TACTCTGATGTTTTATATCTTTTCTAA |
| <i>BC2215-fw</i> | CCATTTAAATACGTCGGTGT |
| <i>BC2218-rv</i> | ATATGTTTCGGCAATACTTCG |
| <i>5Up-5412-11</i> | TCGATGCATGCCATGGACCTGAAAAGGTGGTAAATC |
| <i>3Up-5412-11</i> | TTGTCTACAGATTAACTCTTGCTAACCGATTTCCT |
| <i>5Dn-5412-11</i> | TCACGGTTTACCCACAGAAACAAATCCCGATGTTA |
| <i>3Dn-5412-11</i> | GGGCGATATCGGATCCTTGCGTACGATCAGATAAAT |
| <i>5spcR</i> | TTAATCTGTAGACAAATTGTGA |
| <i>3spcR</i> | GTGGGTAAACCGTGAATATC |
| <i>Spc5out</i> | TCACAATTTGTCTACAGATTAA |
| <i>Spc3out</i> | GATATTCACGGTTTACCCAC |
| <i>Cpl2216-17-Fw-Sal</i> | GACGCG <u>TGCAGC</u> AGGTAGAGCAGACGATTCAA |
| <i>Cpl2216-17-Rv-Bam</i> | GCGGG <u>ATCCTTT</u> ATCCTGTAAAAACCGATTA |
| <i>qPCR-BC2216-F</i> | AAAAGAGATGCGTTCGTTTAG |
| <i>qPCR-BC2216-R</i> | ACACACCAACAGTTTCCTTC |
| <i>qPCR-16S-F</i> | GGTAGTCCACGCCGTAAACG |
| <i>qPCR-16S-R</i> | GACAACCATGCACCACCTG |

^a Restriction enzyme sites are underlined

Mutant construction

The BC_2216 and BC_2217 genes encoding a putative histidine kinase and a cognate response regulator, respectively, were interrupted in *B. cereus* ATCC 14579 by allelic exchange with a cassette conferring kanamycin resistance (Km^r), as previously described (Arnaud et al., 2004b). Briefly, DNA fragments of the BC_2216 upstream region and the BC_2217 downstream region were PCR-amplified using the primer pairs 5Up-2216-17-Eco/3Up-2216-17-Pst and 5Dn-2216-17-Sal/3Dn-2216-17-Bam, respectively (Table 2). PCR products were digested with *EcoRI/PstI* and *SalI/BamHI* using the primer-incorporated

restriction sites (Table 2). In parallel, the Km^r cassette (1.5 kb fragment corresponding to the *aphA3* kanamycin resistance gene with its own promoter) was digested from pHT304-Km (Table 1) with *PstI/SalI*. The three digested DNA fragments were purified, ligated in *EcoRI/BamHI*-digested pMAD (Table 1), and introduced by electroporation in *E. coli* TG1. Unmethylated plasmids were then prepared from *E. coli* ET 12567 and the resulting recombinant plasmid pMAD Δ BC_2216-17 was transformed into *B. cereus* ATCC 14579. Transformants were then subjected to allelic exchange as previously described (Arnaud et al., 2004b). Colonies that were resistant to Km and sensitive to Em arose through a double-crossover event in which the chromosomal wt copies of BC_2216 and BC_2217 genes were deleted and replaced by the Km^r cassette. Chromosomal allele exchange in the mutant was checked by PCR using the appropriate primer pairs (BC2215-Fw/Km5out and Km3out /BC2218-Rv, see Table 2). PCR products were sequenced for confirmation.

Similarly, the BC_5412 and BC_5411 genes encoding putative paralogs of BC_2216 and BC_2217 genes were interrupted in *B. cereus* ATCC 14579. First, the SpR cassette, a 1.2 kb fragment containing the *spc* spectinomycin resistance gene with its own promoter (Murphy, 1985) was PCR amplified from pDIA (obtained from I. Martin-Verstraete (Laouami et al., 2011)) using primer pairs 5spcR and 3spcR (Table 2). In parallel, primer couples 5Up-5411-12 /3Up-5411-12 and 5Dn-5411-12 /3Dn-5411-12 (Table 2), harbouring DNA sequence overlapping the SpR cassette or the pMAD vector, were used to amplify upstream and downstream regions from BC_5412-BC_5411 genes. These two PCR fragments were purified and mixed with both the SpR cassette and the *BamHI/NcoI*-digested pMAD, to be ligated altogether using the InFusion HD cloning kit (Clontech, Mountain view, CA, USA) according to the manufacturer's instructions. The mixture was transformed in *E. coli* as recommended by the manufacturer. The appropriate plasmid was isolated and checked by DNA sequencing of the insert region. It was then transformed into *B. cereus* ATCC 14579 and subjected to allelic exchange, as described above.

Complementation in *trans* of the Δ BC_2216-17 mutant was performed by introducing both the BC_2216 and BC_2217 genes with a large upstream region (889 bp upstream from ATG) presumably containing their own promoter, on a pHT304 plasmid (Table 1). This 2848 bp DNA region was obtained by PCR amplification using the primers Cpl2216-17-Fw-Sal and Cpl2216-17-Rv-Bam (Table 2).

In addition to the ATCC 14579 model strain (group IV), we selected three other strains from distinct phylogenetic groups and with available genome sequences, i.e. AH187 (group III),

MM3 (group II) and Rock 3-28 (group V). Their ability to grow at low temperature, particularly the minimal temperature for growth (T_{min}), was checked as described in Bergey's Manual (Claus and Berkeley, 1986). The studied strains showed different degrees of temperature tolerance, from psychrotolerance with a T_{min} of 7°C (MM3) to moderate psychrotolerance with a T_{min} of 8°C (Rock 3-28) and mesophilic ability with T_{mins} of 10°C (ATCC 14579) and 12°C (AH187).

Transformation by electroporation into of AH187, MM3 and Rock 3-28 strains was performed as described above using the recombinant plasmid pMAD Δ BC_2216-17 (Table 1) isolated from the ATCC 14579 strain. The protocol leading to disruption of the desired genes by a double cross-over was then realized as described above for the ATCC 14579 strain, using the following modifications for the two psychrotolerant strains (MM3 and Rock 3-28). Successive cultures were performed with a gradual increase (by steps of 2°C or 4°C) of the incubation temperature from 30°C up to 37°C for MM3 or up to 40°C for Rock3-28 (these strains are unable to grow at temperatures above 40°C) in order to allow conditions in which the thermosensitive plasmid could be lost following a recombination event. When Km-resistant and Em-sensitive colonies arose through a double-crossover event, we checked for correct chromosomal allele exchange by PCR as described above for the ATCC 14579 strain.

***In silico* analysis**

Affiliation to phylogenetic groups for the strains used in this study and for the 196 *B. cereus* genomes that were available in databases at the time of the search (November 2013) was established as previously described (Guinebretiere et al., 2010), using *panC* sequence similarity. The search for BC_2216 and BC_2217 orthologs was performed via the IMG (Integrated Microbial Genomes) interface (Markowitz et al., 2012). In a first step, candidate homologs are identified based on BLASTp similarities with 1e-2 E-value cutoff and low complexity soft masking (-F'm S') turned on. In a second step, ortholog relationship between BC_2216 and BC_2217 genes and homolog genes in all other genomes is established through bidirectional best hits. Because paralogs were present in many strains (BC_5411 and BC_5412 in ATCC 14579), we considered genuine BC_2216-BC_2217 orthologs when orthologs of the surrounding genes were present with conserved synteny. To achieve this, gene neighborhoods in all available genomes were compared using the Conserved Neighborhood Viewer bundled with the IMG interface (Markowitz et al., 2006). Protein

domains in BC_2216, BC_2217, BC_5412 and BC_5411 were identified using SMART (Letunic et al., 2012, Schultz et al., 1998).

Results

The BC_2216 and BC_2217 genes encode a 2CS and are overexpressed at low temperature.

According to their annotation, the BC_2216 and BC_2217 genes found in the *B. cereus* ATCC 14579 mesophilic strain, respectively encode putative histidine kinase and response regulator. The predicted BC_2216 protein indeed contains the classical transmitter domains of histidine kinases: an ATP binding domain (HATPase domain, residues 288-372), and a dimerization and phosphoacceptor domain (PFAM:HisKA_3 domain, residues 186-247). BC_2216 has no predicted transmembrane domain but its N-terminal region displays a GAF domain (Pfam 01590). BC_2217, the presumed cognate response regulator of BC_2216, displays a phosphoacceptor site (REC domain, residues 6-120) and a DNA-binding motif (HTH domain, residues 151-207), suggesting that BC_2217 has DNA regulatory ability. The BC_2216 and BC_2217 proteins thus presumably form a 2CS.

The level of expression of the BC_2216 gene was quantified by RT-qPCR during low-temperature growth (12°C) and compared to that at an optimal temperature (37°C). RNA samples were extracted from cells collected at three times during growth kinetics, in mid-exponential phase (OD₆₀₀ = 0.5), end-exponential phase (OD₆₀₀ = 1.0) or stationary phase (OD₆₀₀ = 2.5). Results showed 7.2-fold, 10.4-fold and 9.7-fold overexpression, respectively, during growth at 12°C compared to 37°C, suggesting an important role of the BC_2216-BC_2217 2CS during this growth condition.

Mutation of the BC_2216 and BC_2217 genes causes impaired low-temperature growth.

A BC_2216-BC_2217 mutant was constructed in the *B. cereus* ATCC 14579 strain by allelic exchange between the two 2CS encoding genes and a kanamycin resistance cassette (Table 1), and the phenotype of this mutant was compared to that of its parental strain during growth at various temperatures (Fig. 1).

At the T_{min} of this strain (i.e. 10°C), the wt growth curves varied slightly between experiments. The lag phase of the 3 replicates was about 114 ± 14h (mean ± sem) and they reached stationary phase in 196 ± 21 h after inoculation, with a maximal OD of 2.3 ± 0.1. In

the same conditions, the BC_2216-BC_2217 mutant did not show any growth, even after 300 h of incubation.

At 12°C, the mutant was able to grow but the lag phases of the wt (91.3 ± 6.9 h) and the mutant strains (189.3 ± 6.7 h) from three independent cultures were significantly different (mean \pm SEM, $p < 0.05$, student t-test), indicating that the mutant was slower to adapt to this cold temperature than the wt strain. The maximum OD was also significantly lower for the mutant than for the wt (3.68 ± 0.10 vs 4.58 ± 0.19 , respectively, $p < 0.05$). Thus, mutant growth was impaired at 12°C.

In contrast, at optimal temperature (i.e. 37°C), the growth curve of the two strains were similar. The cells reached the stationary phase with only a slight delay of 1.19 h ($p < 0.05$) for the mutant compared to the wt strain, but maximal ODs were not significantly different (2.01 ± 0.09 for the mutant vs 2.06 ± 0.01 for the wt, $p > 0.05$).

Microscopic observations were performed during growth at low and optimal temperature (Fig. 2). Both wt and mutant strains showed slight cell elongation during growth at 12°C compared to growth at 37°C (Fig. 2A). There was no difference in cell sizes between wt and mutant strains. Cells of the two strains checked at a higher magnification by TEM showed a similar cell structure at the optimal growth temperature (i.e. 37°C) (Fig. 2B). In contrast, when incubated at low temperature (12°C), cells of the mutant strain displayed an atypical morphology compared to the wt strain, as their cytoplasm displayed a "wavy" shape, suggesting an irregular thickness of the cell wall.

Given that cold is a major stressor for bacteria, we investigated whether the growth of the BC_2216-BC_2217 mutant was impaired when encountering other stressful conditions. Growth of both strains was measured in various conditions: high temperature, low pH, high pH, high osmolarity, and in presence of ethanol, ions chelators, or oxidative agents. In all the tested conditions, the BC_2216-BC_2217 mutant strain showed similar growth to wt (Table S1).

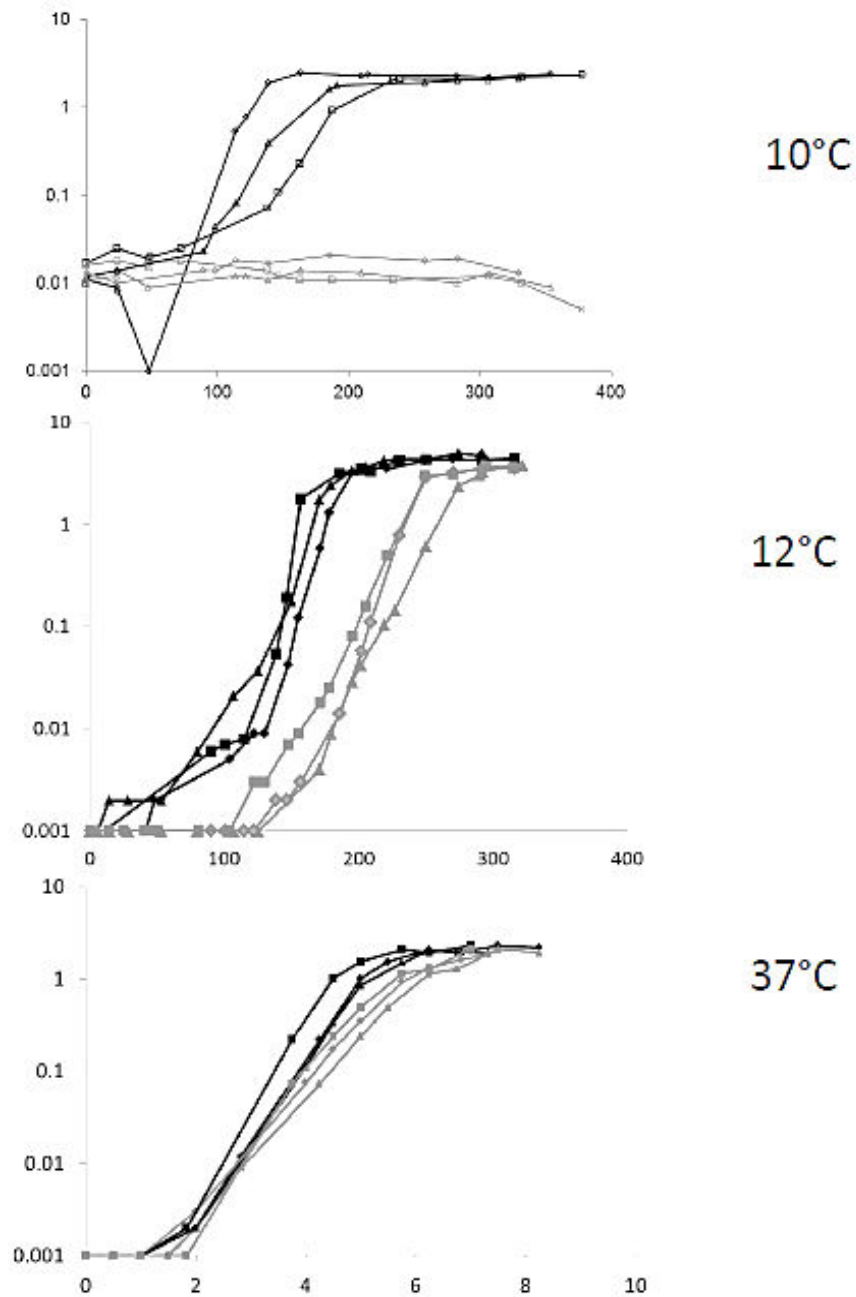


FIG 1 Growth curves of *B. cereus* wt and $\Delta BC_{2216-17}$ strains at various temperatures. Growth of the wt (black) and $\Delta BC_{2216-17}$ (gray) strains was performed in 100 ml LB at the indicated temperature under shaking. Three biological replicates are shown for each condition (different symbols represent different replicates). During the lag phase at 10°C, cells sometimes formed temporary small aggregates which caused a decrease in the measured OD. Y axes indicate the ODs at 600 nm; x axes indicate times (in hours).

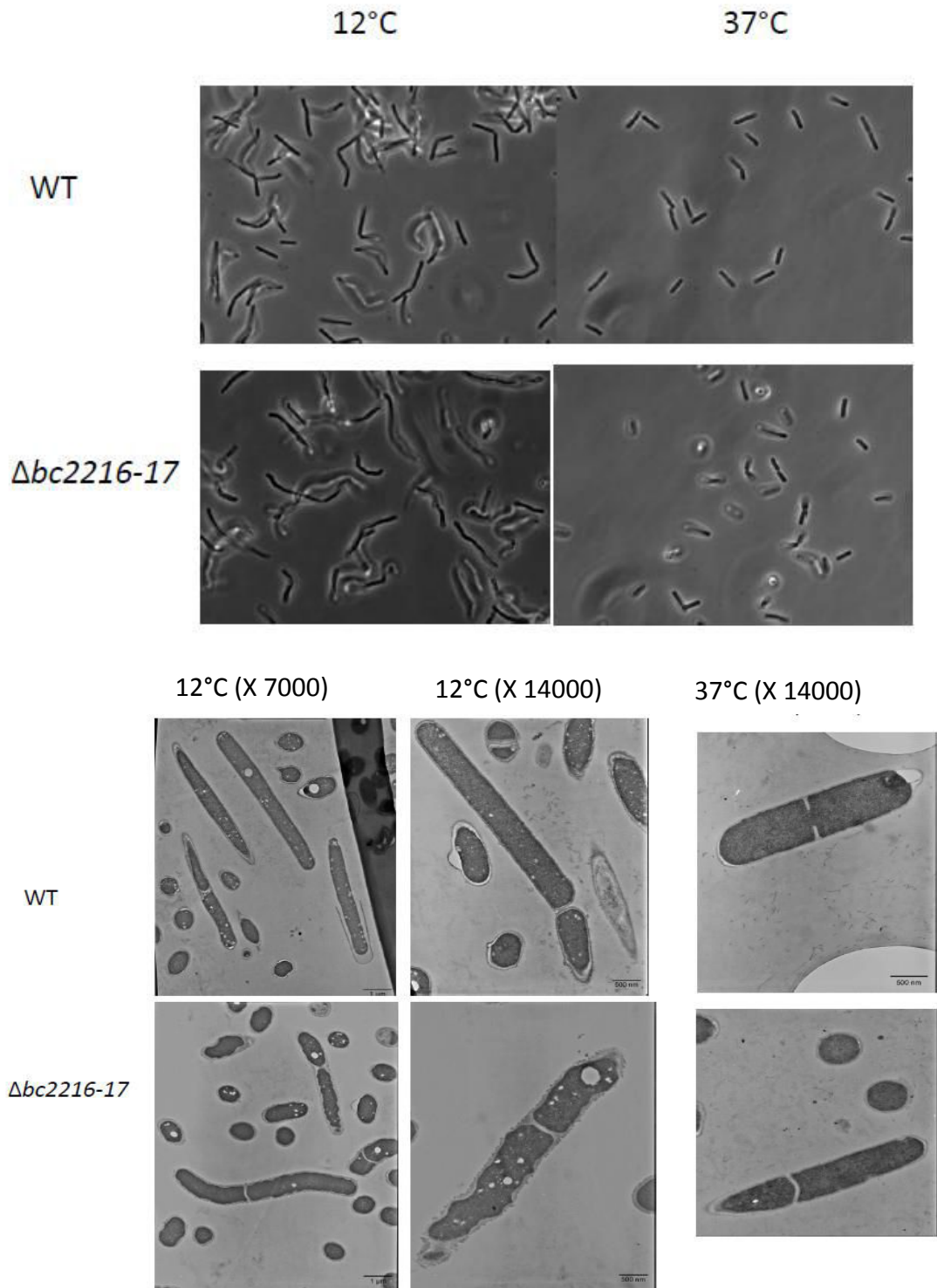


FIG 2 Microscopic observations of *B. cereus* wt and *_BC_2216-17* strains grown at 12 and 37°C until the stationary phase. Microscopic observations were made under a phase-contrast microscope at a magnification of $\times 1,000$ (A), and under a TEM at magnifications of $\times 7,000$ and $\times 14,000$ (B).

In addition, the phenotype of the mutant was compared to that of its parental strain after growth on various media. Growth on Mossel and growth on sheep blood agar showed that the mutant and the wt strains displayed the same lecithinase activity and hemolytic profile, respectively. Tests run on an API50CH kit to determine the bacterial oxidation of 49 different carbohydrates did not find any differences between the two strains.

Taken together, these data suggest that the major phenotype of this mutant, compared to that of the wt, was its growth impairment at low temperature. We therefore propose to name *casK*, for cold adaptation sensor, the BC_2216 gene that encodes a putative histidine kinase, and *casR* the BC_2217 gene that encodes a putative response regulator.

Survival at temperatures below the minimal temperature of growth

Given the impaired growth at low temperature, we investigated the ability of ATCC 14579 wt and \square *casKR* to survive at temperatures below the minimal temperature of growth (i.e. below 10°C). The two strains were incubated at 4°C in LB, and CFU were enumerated over time. As shown in Fig. 3, viable counts of both strains decreased regularly over time during incubation at 4°C, but the viability loss was significantly faster for the *casKR* mutant than for the wt strain. A viability loss of 3 log CFU was reached in 103 ± 6 h (mean \pm SEM) for the mutant strain vs 292 ± 46 h for the wt ($p < 0.05$, student t-test). Thus, after 300 h of incubation, the viable counts were significantly higher for the wt (4.7 ± 0.6 log CFU, mean \pm SEM) than for the *casKR* mutant (2.5 ± 0.3 log CFU, $p < 0.05$).

In order to determine whether long-term storage in LB medium at 37°C could also impair survival the same way, we ran a control experiment. Results indicate that viability loss was very limited, with a 1.1 log loss for the ATCC 14579 wt vs a 1.3 log loss for the *casKR* mutant (means of duplicate experiments) after 400 h of incubation at 37°C (Fig. S2). This result indicates that the ability to survive long-term storage in LB involves CasKR only at low temperature.

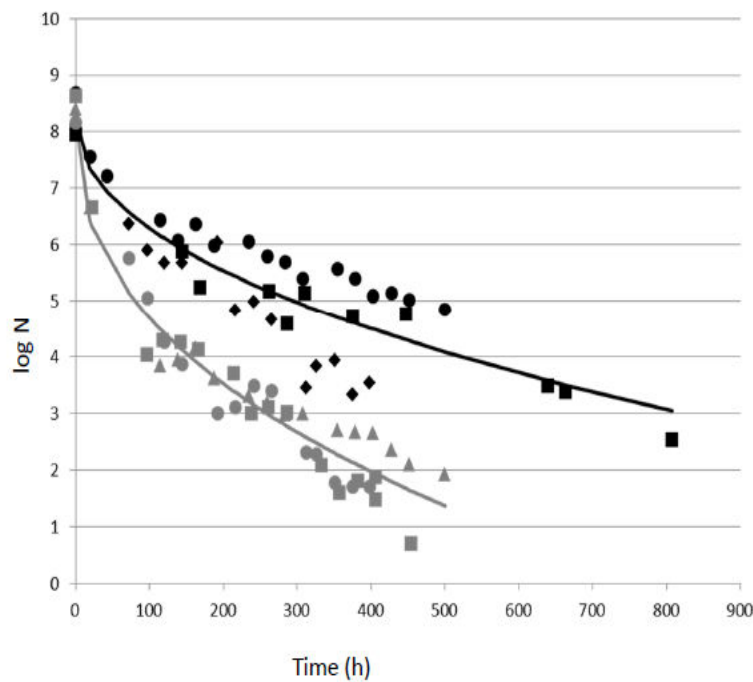


FIG 3 Survival of *B. cereus* wt and $\Delta casKR$ strains at 4°C. *B. cereus* wt (black symbols) and $\Delta casKR$ (gray symbols) strains in bacterial suspensions were incubated in LB at 4°C. CFU enumeration was performed every day. Different symbols represent data from 3 different replicates. The curves represent theoretical data calculated as described in Materials and Methods.

Complementation of the mutant phenotype

Complementation of the $\Delta casKR$ strain was performed by introducing on a plasmid the *casKR* genes and a large upstream region presumably containing the *casKR* promoter (Table 1). While no growth was observed for the $\Delta casKR$ strain without complementation at the T_{min} (i.e. 10°C), growth ability was restored for the complemented strain $\Delta casKR + pHT-casKR$ (Fig. 4). CFU were detected on LB agar even at a 10^{-7} dilution, similarly to what was observed for the ATCC 14579 wt strain (Fig. 4). This result confirms that the *casKR* deletion is genuinely responsible for the cold growth impairment of the mutant strain.

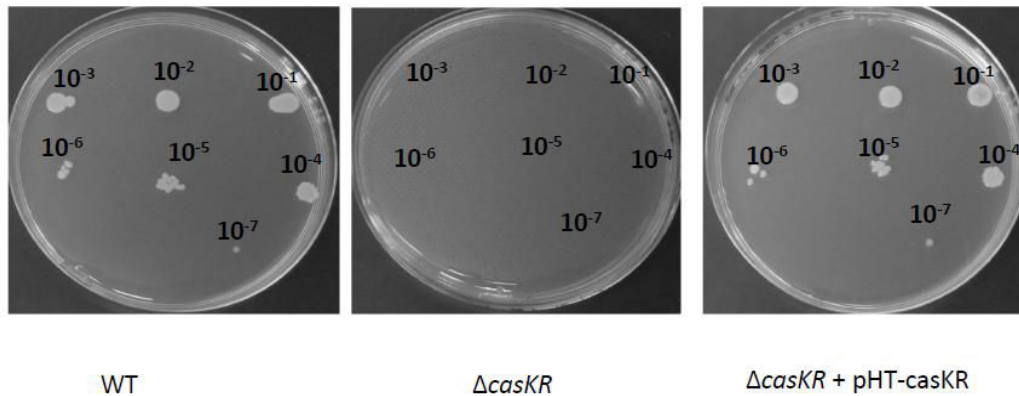


FIG 4 Complementation of $\Delta casKR$ restores the ability to grow at low temperature. Five microliters of serial dilutions of the *B. cereus* wt, $\Delta casKR$, and $\Delta casKR/pHT-casKR$ strains (grown in liquid medium at 30°C and adjusted to 108 CFU/ml) was spotted on LB agar, and the strains were incubated at 10°C for 20 days.

The CasKR paralog is not required for *B. cereus* low-temperature growth

The *B. cereus* ATCC 14579 BC_5412 and BC_5411 genes present high sequence similarity with the *casK* and *casR* genes located elsewhere on the chromosome (49.5% and 54.0% identity, respectively). Consequently, BC_5412-BC_5411 2CS could be considered as a paralog of CasKR. We therefore investigated whether BC_5412-BC_5411 could play a role similar to CasKR during *B. cereus* growth at low temperature. A BC_5412-BC_5411 mutant was constructed and its growth was tested at optimal and at low temperature. There was no observable difference between the BC_5412-BC_5411 mutant and its parental strain in terms of kinetics of growth at 37°C (data not shown) or 12°C (Fig. S3). At T_{min} (i.e. 10°C), this mutant was still able to grow on LB agar similarly to the wt (Fig. S3).

Role of CasKR among other *B. cereus sl* strains

To determine whether the *casKR* genes are present among other strains of the various phylogenetic groups of *B. cereus sl*, we performed a search for *casKR* (BC_2216-BC_2217) orthologs with conserved synteny of gene neighborhoods (Fig. S4). Sequence similarity and e-values are reported for each ortholog in Table S5. Orthologs of *casKR* were found in strains belonging to five of the seven known phylogenetic groups of *B. cereus sl*, i.e. groups II to VI (Table 3, Fig. S4, Table S5). While absent in group VII (a moderate thermotolerant group), *casKR* orthologs were present in mesophilic group I, but synteny was not conserved, particularly for downstream genes.

Table 3. Search for *casKR* orthologs among the 7 phylogenetic groups of *B. cereus sl*.

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| Phylogenetic group | Orthologs of BC_2215, BC_2218 | | | | Psychrotolerant strains | Human pathogenic strains | Strain used for construction of the <i>casKR</i> mutant during this study |
|--------------------|-------------------------------|----------------|----------------|---------|-------------------------|--------------------------|---|
| | BC_2215 | BC_2216 | BC_2217 | BC_2218 | | | |
| I | - | + | + | - | - | - | (ND) |
| II | + | + | + | + | + | + | MM3 |
| III | + | + | + | - | - | + | AH187 |
| IV | + | + | + | + | - | + | ATCC 14579 |
| V | + | + | + | + | + | + | Rock 3-28 |
| VI | + | + | + | + | + | - | (ND) |
| VII | - | - | - | + | - | + | (NA) |

An *in silico* BLASTp search was performed to screen for orthologs of the BC_2216-BC_2217 genes among 196 *B. cereus* strains of the various phylogenetic groups (Guinebretiere et al., 2008a) for which genomic data were available. A plus sign indicates that BC_2216 and BC_2217 orthologs (in bold) in addition to BC_2215 or BC_2218 orthologs were found with a conserved synteny. A minus sign indicates an absence of the ortholog or synteny not conserved. Among each of the seven phylogenetic groups, presence of human pathogenic strains and psychrotolerant strains is indicated as previously described (Guinebretiere et al., 2008a, Guinebretiere et al., 2010). CasKR mutants were constructed during this study in strains belonging to 4 of the 7 phylogenetic groups, as indicated. ND, not done; NA, not applicable.

Outside *B. cereus sl*, tBlastN analysis revealed that the best similarity was found in *B. megaterium* (51% identity for *casK* and 62% for *casR*) but synteny was not conserved. In other species, similarity was lower and the synteny was still not conserved. For instance, in *B. subtilis* strain 168, the best similarity was obtained with *yhcYZ*, a 2CS of unknown function (48% identity for *casK* and 47% for *casR*). These genes, found outside *B. cereus sl* strains, may be considered as putative *casKR* orthologs, but their function remains to be investigated to clarify this point.

Thus, the *casKR* genes are widespread among *B. cereus sl* strains. Psychrotolerant and moderate psychrotolerant strains are clustered in 3 out of 7 phylogenetic groups of *B. cereus sl* (Guinebretiere et al., 2008a) and they all displayed orthologs of *casKR* genes. We thus investigated whether the role of these orthologs was similar to that observed in the mesophilic ATCC 14579 strain (group IV), despite a lower T_{min} for these strains. Orthologs of the *casKR* genes were also found among strains belonging to phylogenetic group III which are

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considered to have a higher T_{min} (12°C) than the ATCC 14579 strain (10°C). We therefore investigated the role played by *casKR* in 3 strains representative of 3 additional phylogenetic groups by constructing a *casKR* mutant for each strain.

For the mesophilic AH187 strain (phylogenetic group III), the psychrotolerant MM3 strain (group II) and the psychrotolerant Rock 3-28 strain (group V) studied, the *casKR* mutants and their parental strains displayed similar growth ability at 37°C (Fig 5). In contrast, all 3 *casKR* mutants showed impaired growth at low temperature. In addition, the mutants of the two psychrotolerant strains did not show any growth at 8°C, a temperature close to their T_{min} (i.e. 8°C for the Rock 3-28 strain and 7°C for the MM3 strain).

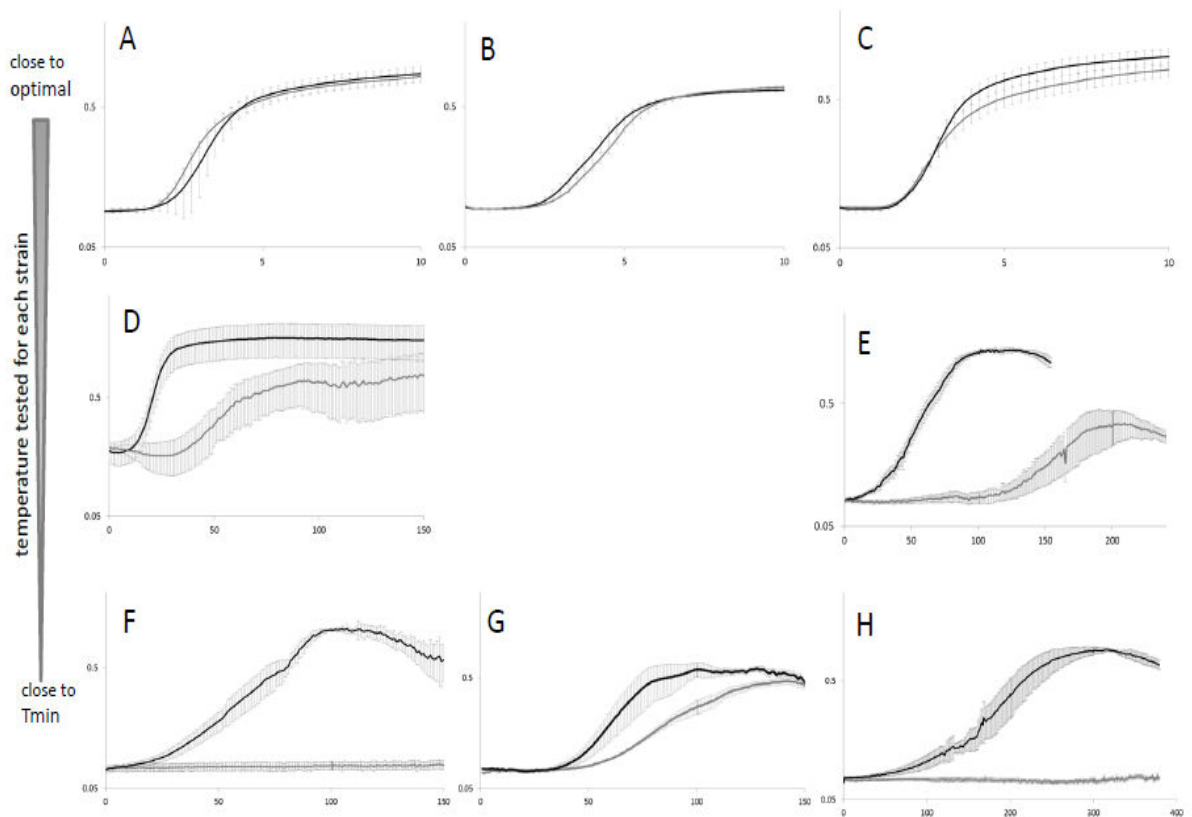


FIG 5 Growth at various temperatures of wt and $\Delta casKR$ strains from phylogenetic groups II, III, and V of *B. cereus sensu lato*. Growth of wt and $\Delta casKR$ strains belonging to phylogenetic group II (strain MM3) (A, D, F), group III (strain AH187) (B, G), and group V (strain Rock 3-28) (C, E, H) was performed in an automated turbidimeter with shaking at 37°C (A to C), at a low temperature of 13°C (D) or 10°C (E), or at a temperature close to the T_{min} of the strains of 8°C (F, H) or 12°C (G). y axes indicate the OD at 600 nm; x axes indicate time (in hours). Mean values \pm SDs of three biological replicates are shown for each condition. Black curves, wt strains; gray curves, $\Delta casKR$ mutants.

These results showed that the CasKR 2CS was required for growth at low temperature among *B. cereus sl* in mesophilic as well as in psychrotolerant strains, whatever the value of the

T_{min}. In addition, CasKR seems to be particularly efficient when the bacteria are submitted to a temperature close to their T_{min}.

Discussion

In this study, we identified a pair of genes that play a major role in *B. cereus* growth and survival at low temperature. CasKR, the newly identified 2CS, is widespread among *B. cereus* *sl.* 2CS have a wide array of functions and a few 2CS have already been shown to be involved in low-temperature response in other bacteria (DesKR in *B. subtilis*, CheAY in *Y. pseudotuberculosis*, CBO0365-0366 in *C. botulinum*) (Aguilar et al., 2001c, Dahlsten et al., 2014b, Lindstrom et al., 2012, Palonen et al., 2011). According to the proposed 2CS classification scheme (Fabret et al., 1999a, Mizuno, 1997b), CasK belongs to the class II family of histidine-kinase and CasR belongs to the NarL family of cognate response regulators, which puts CasKR in the same family as DesKR. However, despite their signal transduction function, these two 2CS have completely different genetic organizations (for instance, the desaturase-encoding genes are located on different loci in *B. cereus*, contrary to what is observed in *B. subtilis*) and the two HK display no similarity within their N-terminal sensory domains. Among the various 2CS present in the ATCC 14579 model strain of *B. cereus*, CasKR (the BC_2216-BC_2217 2CS) and its paralog BC_5412-BC_5411 2CS seem atypical as their kinase does not display a transmembrane domain, suggesting that they belong to those rare HK that have a cytoplasmic location (Krell et al., 2009b). Despite the sequence similarity between these two paralogs, only CasKR is overexpressed at low temperature (Chamot & Brillard, unpublished results) and only CasKR is necessary for optimal growth at low temperature. According to the SMART analysis, the sensory N-terminal region of CasK contains a GAF domain (Schultz et al., 1998). These domains are able to bind cyclic nucleotides and are present for instance in cGMP-specific phosphodiesterases or in phytochromes (Ho et al., 2000a). To our knowledge, a link between such a domain and low-temperature-sensing has never been described. This raises the question of the signal perceived by CasK in cold conditions. A 2CS from *Staphylococcus aureus*, YhcSR, presents some sequence similarity with CasKR. As YhcS lacks a transmembrane domain, this histidine kinase probably has an intracytoplasmic location, like CasK (Sun et al., 2005). This 2CS was shown to be essential for cell viability, and it controls the expression of an ABC transporter that seems to play a role in high osmotic conditions (Yan et al., 2012). The full viability of our

CasKR mutant and its unaffected growth in high NaCl concentrations compared to the wt suggest that YhcSR and CasKR play different roles in cell physiology.

Whatever the tested *B. cereus sl* strains among several phylogenetic groups, $\Delta casKR$ mutants showed impaired growth at low temperature compared to their parental strains. In ATCC 14579, survival at 4°C was also impaired in the $\Delta casKR$ mutant compared to that of the wt. In contrast, the wt and the mutant showed similar survival at sub-freezing temperature (-20°C) (Diomande & Brillard, unpublished data). These results suggest a major role of CasKR when cells are physiologically active (10°C) or when metabolic activity is strongly reduced (at 4°C), but not when cells are frozen.

Another phenotype of the CasKR mutant is the modified cell shape with an irregular cell wall instead of the elongated rod-shaped cells regularly observed for the wt at low temperature. Strong modifications of the structures of *B. cereus* cells have been described before, for instance in a *csxA* RNA-helicase mutant with a substantially stronger phenotype (Pandiani et al., 2010b). In another study in conditions involving both low temperature and low redox potential, elongation of the cells coupled to a default in cell separation was observed (de Sarrau et al., 2013b).

Model strains are good tools to identify new mechanisms, but they may phenotypically differ significantly from food-poisoning strains (Muniesa et al., 2012), which may also be true for *B. cereus* (Brillard and Lereclus, 2004, Fagerlund et al., 2007, Lapidus et al., 2008b). *B. cereus sl* has the singularity of displaying a wide panel of strains: some are pathogenic and mainly belong to mesophilic or thermotolerant groups III, IV and VII, others belong to psychrotolerant group II and V where fewer pathogenic strains have been so far described, and the most psychrotolerant strains from group VI have only marginally been associated to human pathogenicity (Guinebretiere et al., 2010, Thorsen et al., 2006a). Therefore, the ability of some *B. cereus sl* strains to both grow at low temperature and to cause human infections or food poisoning makes it important to identify the mechanisms involved in cold growth of both mesophilic and psychrotolerant strains. The CasKR 2CS has been identified in various strains belonging to phylogenetic groups II, III, IV, V and VI, and we proved its involvement in low-temperature growth in strains representative of phylogenetic groups II, III, IV and V, two of which are classified as psychrotolerant and two as mesophilic. It is tempting to speculate that CasKR could also play a similar role in psychrotolerant strains belonging to phylogenetic group VI (*B. weihenstephanensis*), but this was not investigated here because several attempts to obtain a *casKR* mutant in 2 different strains from this group failed. Such unsuccessful

assays could reveal a major role of CasKR in these strains, but clarification of this point will have to be deciphered by further investigations. Excluding the AH187 emetic strain (Ehling-Schulz et al., 2005), the strains chosen here were not isolated from a food-poisoning outbreak. However, all these strains have the pathogenic potential to cause foodborne illness, as their genome contains genes involved in virulence (eg. *nhe*, *plcR*) (Salamitou et al., 2000, Stenfors Arnesen et al., 2008c). Our results illustrate that psychrotolerant *B. cereus sl* strains could require CasKR to achieve efficient growth during food storage prior a possible food poisoning event.

Thus, CasKR seems to be a dedicated 2CS among *B. cereus sl* that could participate in the low-temperature adaptation of both mesophilic and psychrotolerant strains despite their different ranges of growth temperature. Interestingly, *casKR* orthologs are absent from the genome of strains of phylogenetic group VII, i.e. the most thermotolerant strains of *B. cereus sl*. Through this coincidence, it could be assumed that the lack of *casKR* genes might be linked to the inability of strains from this group to grow at temperatures below 18°C. However, these strains also probably lack many of the tools needed for low-temperature growth, given that they have a fairly smaller genome than other *B. cereus sl* strains (Lapidus et al., 2008b). Although the chromosomal region of *casKR* seems to have been conserved (Fig. S4), these strains are also phylogenetically remote from other *B. cereus sl* strains, and therefore constitute a distinct species (*B. cytotoxicus*) in this group (Guinebretiere et al., 2013b).

Conclusion

Food safety issues caused by *B. cereus* occur because this foodborne bacterium is able to proliferate in food prior to ingestion by the consumer (Stenfors Arnesen et al., 2008c). Despite refrigeration processes used to limit bacterial proliferation in food, some psychrotolerant strains of *B. cereus* can still grow at these low temperatures. In addition, occasional leaks in the chill chain may also create conditions that allow growth of mesophilic strains of *B. cereus*. Understanding the mechanisms involved in the cold adaptation of the vegetative cells of *B. cereus* may help more accurately estimate the risk of proliferation in food. Efforts to identify such mechanisms have generally been performed in model mesophilic strains (Brillard et al., 2010a, Broussolle et al., 2010b, Pandiani et al., 2010b). Here, we identified CasKR, a 2CS with previously unknown function, which seems to be a general mechanism among *B. cereus sl* that could participate in the low-temperature adaptation of both mesophilic and

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psychrotolerant strains. The detailed mechanism of this 2CS in cold sensing and adaptive response will have to be deciphered by further studies.

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Chapitre 3 :

**CasK/R est nécessaire pour
l'insaturation optimale des acides gras
lors l'adaptation de *Bacillus cereus* au
froid**

Chapitre 3 : CasK/R est nécessaire pour l'insaturation optimale des acides gras lors l'adaptation de *Bacillus cereus* au froid

Chapitre 3 : CasK/R est nécessaire pour l'insaturation optimale des acides gras lors l'adaptation de *Bacillus cereus* au froid

I- Introduction à l'étude

L'adaptation des bactéries au froid est un phénomène connu pour être multifactoriel. Chez *B. cereus*, plusieurs études décrivant comment *B. cereus* s'adapte au froid le montrent aussi (Pandiani et al., 2011a, de Sarrau et al., 2013d, Diomandé et al., 2014, Brillard et al., 2010c, de Sarrau et al., 2012c).

La modulation de la fluidité membranaire représente néanmoins une stratégie cruciale pour la survie des bactéries au froid (Russell, 1984). En effet, la membrane permet les échanges avec le milieu environnemental et la perte de ces propriétés peut être létale pour la bactérie.

La baisse de température altère la fluidité membranaire, la rendant rigide, ainsi les bactéries doivent adapter leur composition membranaire, généralement en utilisant des acides gras à faible point de fusion. Cela suggère donc une diminution de la taille des chaînes des acides gras (AG), l'augmentation du ratio d'AG branchés antéiso/ iso et celle des AG insaturés (Haque and Russell, 2004b, Kaneda, 1977).

Le système à deux composants (TCS) DesK/R de *Bacillus subtilis* est le premier TCS décrit pour son rôle dans l'adaptation au froid, en induisant l'expression d'une désaturase qui va entraîner la modulation de la fluidité membranaire dans cette condition (Aguilar et al., 2001b, Cybulski et al., 2010b).

Chez *B. cereus*, nous avons identifié un TCS, CasK/R dont le rôle dans l'adaptation de *B. cereus* et plus largement de *B. cereus sensu lato* a été démontré (Diomandé et al., 2014). *In silico*, les alignements indiquent 63% d'identité entre *casK* et *desK* et 76% d'identité entre *casR* et *desR*. Mais plusieurs différences existent entre les TCS de ces 2 espèces bactériennes.

Dans ce chapitre nous avons tenté de répondre à plusieurs questions :

-Quels sont les gènes appartenant au régulon de CasR ? Est-il possible d'identifier à partir de ce régulon d'éventuels mécanismes permettant l'adaptation CasK/R-dépendante au froid chez *B. cereus* ?

-Les changements de profils d'AG décrits dans l'adaptation au froid sont-ils différents entre le WT et le mutant $\Delta casK/R$?

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II- Stratégies envisagées

Dans un premier temps, une approche de transcriptomique comparative entre les souches $\Delta casKR$ et WT de *B. cereus* ATCC 14579 a été utilisée, comme décrit précédemment (Brillard et al., 2010c, van Schaik et al., 2007b). Lors de cette étude, des gènes appartenant au métabolisme des acides gras étaient différentiellement exprimés à basse température chez la souche $\Delta casK/R$ par rapport à la souche WT. Nous avons donc étudié les profils d'acides gras (AG) de ces 2 souches à basse température selon la méthode précédemment détaillée (de Sarrau et al., 2013a). L'analyse des profils d'acides gras membranaires a révélé que les AG insaturés (AGI) se sont révélés important pour la croissance de *B. cereus* au froid. Nous avons donc trouvé intéressant d'étudier l'influence que des acides gras insaturés exogènes sur la croissance au froid de la souche $\Delta casK/R$.

III- Résultats et discussion

L'étude transcriptomique réalisée sur la souche modèle et son mutant isogénique $\Delta casK/R$ a révélé une centaine de gènes différentiellement exprimés en début de phase exponentielle et en phase stationnaire, à basse température. Seize de ces gènes, pour la plupart sous-exprimés, codent pour des protéines impliquées dans le métabolisme des AGs, incluant les gènes *desA* et *desB* codant pour les 2 désaturases de *B. cereus*. Ces résultats suggèrent que CasK/R régule le métabolisme des acides gras à basse température.

Les profils d'acides gras réalisés ont ensuite permis de déterminer que les AGI de la membrane sont principalement augmentés lors de la croissance aux basses températures. L'acide gras iso-branchés insaturé iso-C17 :1(5) représentait l'acide gras majoritaire à 12°C chez la souche WT alors qu'à 37°C la proportion de cet acide gras est quasiment 10x fois moins importante.

Lorsqu'on se focalise plus particulièrement sur les AG insaturés par l'action de DesA, on observe une plus faible proportion d'AG insaturés par cette désaturase chez la souche $\Delta casK/R$ par rapport à la souche WT. Aucune différence entre les 2 souches n'est observée pour les AG insaturés par DesB. Tous ces résultats suggèrent que CasK/R joue un rôle dans la modification de la composition membranaire lors de la croissance au froid.

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Pour aller plus loin nous avons voulu vérifier si compléter le milieu en AGI exogènes pouvait permettre de restaurer une croissance optimale chez la souche $\Delta casK/R$ qui possède moins d'AGI que la souche sauvage lors de sa croissance à basse température. L'expérience réalisée en présence de lécithine, une source d'AGI végétal retrouvée dans certains aliments, a permis de restaurer la croissance de la souche mutante au froid, de manière comparable à celle de la souche WT.

Ces résultats permettent de mieux comprendre le mécanisme d'action de CasK/R et mettent en évidence que tout comme DesK/R chez *B. subtilis*, ce TCS régule l'expression d'une désaturase pour l'adaptation au froid de *B. cereus*.

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Involvement of the CasK/R two-component system in optimal unsaturation of the *Bacillus cereus* fatty acids during low-temperature growth

Sara Esther Diomandé^{1,2}, Christophe Nguyen-the^{1,2}, Tjakko Abee^{3,4}, Marcel H. Tempelaars⁴, Véronique Broussolle^{1,2} and Julien Brillard^{1,2}#.

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¹ INRA, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, 84000 Avignon, France

² Université d'Avignon, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, 84000 Avignon, France.

³ Top Institute Food and Nutrition, NieuweKanaal 9A, 6709 PA, Wageningen, The Netherlands.

⁴ Food Microbiology Laboratory, Wageningen University, BornseWeilanden 9, 6708 WG, Wageningen, The Netherlands.

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#Corresponding author: julien.brillard@avignon.inra.fr

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Chapitre 3 : CasK/R est nécessaire pour l'insaturation optimale des acides gras lors l'adaptation de *Bacillus cereus* au froid

Abstract

Bacillus cereus sensu lato comprises a set of ubiquitous strains including human pathogens that can survive a range of food processing conditions, grow in refrigerated food, and sometimes cause food poisoning. We previously identified the two-component system CasK/R that plays a key role in cold adaptation. To better understand the CasK/R-controlled mechanisms that support low-temperature adaptation, we performed a transcriptomic analysis on the ATCC 14579 strain and its isogenic $\Delta casK/R$ mutant grown at 12°C. Several genes involved in fatty acid (FA) metabolism were down-regulated in the mutant, including *desA* and *desB* encoding FA acyl-lipid desaturases that catalyze the formation of a double-bond on the FA chain in positions $\Delta 5$ and $\Delta 10$, respectively. A lower proportion of FAs presumably unsaturated by DesA was observed in the $\Delta casK/R$ strain compared to the parental strain while no difference was found for FAs presumably unsaturated by DesB. Addition of phospholipids from egg yolk lecithin rich in unsaturated FAs, to growth medium, abolished the cold-growth impairment of $\Delta casK/R$ suggesting that exogenous unsaturated FAs can support membrane-level modifications and thus compensate for the decreased production of these FAs in the *B. cereus* $\Delta casK/R$ mutant during growth at low temperature. Our findings indicate that CasK/R is involved in the regulation of FA metabolism, and is necessary for cold adaptation of *B. cereus* unless an exogenous source of unsaturated FAs is available.

Keywords:

B. cereus, TCS, desaturase, cold adaptation, unsaturated FA

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Introduction

Bacillus cereus sensu lato (s.l.) comprises a set of human foodborne pathogens with seven ubiquitous species able to adapt to a wide range of environmental conditions ((Graumann and Marahiel, 1996a, Stenfors Arnesen et al., 2008b). Altogether these bacteria are able to grow from 5°C to 50°C (Lechner et al., 1998, Zhou et al., 2010) and include psychrotolerant, mesophilic and thermotolerant strains (Guinebretière et al., 2008). Moreover, because of their ability to form spores (Kim et al., 2014), *B. cereus* strains are able to survive food processing and may develop in the chill chain or during food refrigeration up to consumption (Stenfors Arnesen et al., 2008a). The upshot is that *B. cereus* is one of the major pathogens commonly encountered in foodborne illness outbreaks (Markland et al., 2013).

Several studies describing the way *B. cereus* adapts to low temperature (Broussolle et al., 2010a, Pandiani et al., 2010a, Brillard et al., 2010c, de Sarrau et al., 2012c, de Sarrau et al., 2013a, Diomandé et al., 2014) have shown that cold adaptation by *B. cereus* is multifactorial (translational factors, membrane modifications...). One key strategy, crucial to the cold survival of bacteria, is the ability to modulate membrane fluidity (Russell, 1984). In addition to physically protecting the cell, the membrane facilitates component exchanges with the environment. Higher membrane fluidity could enable improved transport, thus ensuring osmotic balance and nutrient supply. As low temperatures alter membrane fluidity, bacteria have to adapt membrane composition in order to decrease the melting point of their phospholipids.

Bacterial growth at low temperature causes changes in FA profile, such as decreased FA chain length, and increased of the anteiso/ iso-branched-chain FA and unsaturated FA (UFAs) (Haque and Russell, 2004b, Kaneda, 1977). Membrane components can usually sense the physical stress corresponding to a decrease in temperature and mount a bacterial adaptive response (Mansilla et al., 2003, Chintalapati et al., 2004). *Bacillus subtilis* DesK/R was the first two-component system (TCS) described as involved in cold adaptation. The sensor protein DesK was shown to sense an increase in membrane thickness in response to a decrease in temperature (Aguilar et al., 2001b, Cybulski et al., 2010b). The cognate response regulator DesR thus regulates the expression of a desaturase gene. Desaturases are oxygenases that can remove two hydrogen atoms from a fatty acyl chain and then catalyze the formation of a double bond in the substrate (Sperling et al., 2003). These enzymes use activated

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molecular oxygen and two reducing equivalents for catalysis (Chazarreta-Cifre et al., 2011, Shanklin and Cahoon, 1998). In *B. subtilis*, the *des* gene was found to be responsible for maintaining membrane fluidity during low-temperature growth (Cybulski et al., 2002). In *B. cereus*, two desaturases DesA and DesB were described (Chazarreta Cifre et al., 2013), but no mechanism similar to *B. subtilis* DesK/R has yet been described for members of the *B. cereus* group.

We previously described a TCS named CasK/R involved in cold adaptation of *B. cereus sensu lato* (Diomandé et al., 2014). Here, we show that CasK/R plays a role in regulating FA composition at low temperature and that lecithine, an exogenous UFAs source sometimes found in food, can be used by *B. cereus* to restore its growth ability at low temperature in absence of CasK/R.

Material and methods

Strains and growth conditions

B. cereus strains used in this study were ATCC 14579 and $\Delta casKR$, its isogenic *casK/R* mutant (Diomandé et al., 2014). They were grown in Luria broth (LB) medium or AOAC medium as previously described (Song et al., 2012b) with vigorous agitation at 37°C, 13°C or 12°C. As required, kanamycin concentration used for bacterial selection was: 150 $\mu\text{g mL}^{-1}$

Transcriptome analysis

Cultures of *B. cereus* ATCC 14579 WT and $\Delta casK/R$ strains were grown in LB medium at 12°C with shaking. As WT and $\Delta casK/R$ strains displayed different growths at 12°C, we standardized the culture samples by harvesting at entry into exponential phase (EP) when cultures rose from an initial $\text{OD}_{600\text{nm}}=0.001$ to reach $\text{OD}_{600\text{nm}}=0.006$ (monitored on the spectrophotometer Helios Epsilon, Thermo scientific) and at entry into stationary phase (SP) when the cultures reached $\text{OD}_{600\text{nm}}=3.4$.

Total RNA were extracted as follows: cells from 100 mL (EP) or 2 mL (SP) of culture were centrifuged at $8,000 \times g$ for 3 min at 4°C and the pellets were immediately frozen at -80°C in TRI-Reagent (Ambion, Huntingdon, United Kingdom) as described before (Brillard et al., 2010c).

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Cell disruption was performed by adding 0.3 g of sterile 0.1 mm zirconium beads (VWR) followed by 3 runs of 30 sec at a speed 6 on a FastPrep Instrument (FP120). The RNAs were then phenol-chloroform-extracted.

cDNA were generated on 20 µg of RNA isolated from the WT and the $\Delta casK/R$ strains as described before (van Schaik et al., 2007a). After purification, cDNAs were labeled with Cy3 and Cy5. The combined cDNAs of WT and $\Delta casK/R$ strains were hybridized onto a 70-mer oligonucleotide micro-array covering all 5,352 open reading frames of the *B. cereus* ATCC 14579 genome. The microarrays were hybridized with 300 ng labeled cDNA for each sample. After hybridization at 60°C for 17 hours, the microarrays were washed with 6x SSC-0.005% Triton X-102 (10 min, 20°C), 0.1x SSC-0.005% Triton X-102 (10 min, 20°C) and 0.1x SSC-0.005% Triton X-102 (10 min, 37°C). Slides were scanned with an Agilent microarray scanner (G2565BA) (extended dynamic range scan mode) and data were processed as described before (van Schaik et al., 2007a) using the web-based VAMPIRE platform (P-value threshold < 0.05) (Hsiao et al., 2005). All experiments were performed with two biological duplicates (including Cy3/Cy5 dye-swaps).

Fatty acid (FA) composition

FAs methyl esters were prepared as previously described (Brillard et al., 2010c, de Sarrau et al., 2013d). Culture samples were harvested in stationary phase (as described above). A volume of 50 mL of cultures was collected. Samples were washed once with a saline solution (0.9% NaCl w/v), except for cultures with exogenous-source FA which were washed three times. The FA obtained from 60 mg of cells (fresh weight) were transesterified by the ester link method (Schutter and Dick, 2000). The reaction consisted of alkaline methanolysis breaking the ester link in the lipid and producing FA methyl esters by reaction with 5 mL of 0.2 M KOH in methanol at 37°C for 1 h. One mL of 1 M acetic acid was then added to lower pH to 7.0. pH was checked with pH test strips. FA methyl esters were then extracted by adding 3 mL of hexane. The supernatant (apolar phase) was transferred into clean tubes and concentrated by evaporation at room temperature under a continuous nitrogen flow to obtain approximately 200 µL of extract. The extracts were injected into a gas chromatography-mass spectrometry (GC-MS) system (Shimadzu QP 2010-01) under the conditions described before (de Sarrau et al., 2013d) and processed by real-time GC-MS analysis.

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Growth with fatty acids sources

Three independent cultures of *B. cereus* ATCC 14579 WT and $\Delta casK/R$ strains were carried out in an automated turbidimeter (Microbiology Bioscreen C Reader, Labsystems, Uxbridge, UK) as previously described (Diomandé et al., 2014). A fresh colony was used to inoculate 10 mL of LB and incubated at 30°C with shaking for 9 h. Ten μL was then used to inoculate 10 mL fresh LB, and incubated at 30°C with shaking for 17 h. This second culture was centrifuged at 4000 rpm, 5 min and the cells were washed once in 10 mL of AOAC medium. The pellet was then resuspended in 10 mL of AOAC medium and this sample was diluted to a concentration of 10^5 CFU/mL in AOAC medium supplemented with 25 $\mu\text{g/mL}$, 125 $\mu\text{g/mL}$ or 375 $\mu\text{g/mL}$ of purified lecithin (Sigma) as a source of UFAs or supplemented with 375 $\mu\text{g/mL}$ of hydrogenated lecithin (Sigma) as a source of saturated FA. Purified lecithin and HPC contains FA of same chain length. Negative controls consisted of the WT and $\Delta casK/R$ strains grown in AOAC without supplementation. Three replicate microplate wells were filled with the dilutions of each inoculated medium to a final volume of 300 μL per well. Cultures were incubated under vigorous constant shaking, and OD_{600} was measured at 1-h intervals at 12°C over a 10-day incubation period.

Results

The *casK/R* mutation affects genes involved in the fatty acids metabolism

A transcriptome analysis was performed on cells that were harvested at two determined growth phase corresponding to the entry into exponential phase (EP) and the entry into stationary phase (SP) (see Materials and methods), taking into account the growth delay of the $\Delta casK/R$ strain at 12°C when compared to the WT strain (Figure 1).

The comparative transcriptome analysis revealed 95 genes down-regulated and 53 up-regulated in the $\Delta casK/R$ strain during both the EP and SP growth phase at low temperature compared to the WT strain (supplementary data Table S1). Among the genes differentially-expressed in both EP and SP, 16 were involved in FA metabolism (Table 1).

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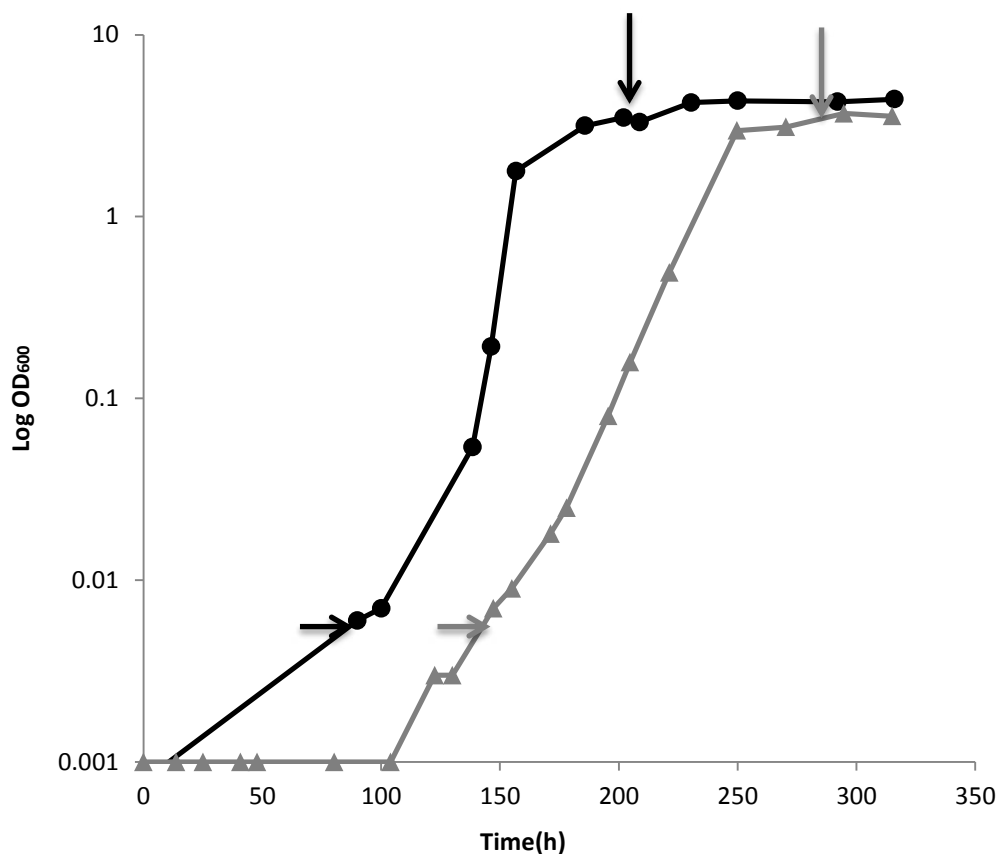


Figure 1: ATCC 14579 WT (black) and $\Delta casK/R$ (grey) growth in LB at 12°C measured by optical density, OD_{600nm}. Arrows indicate sample collection for transcriptomic analysis. Curves shown are representative of WT and $\Delta casK/R$ duplicate growth at 12°C.

Six down-regulated genes were putatively involved in the FA biosynthesis pathway. Among them, *fabH*, *fabF* and *fabI* are involved in saturated FA synthesis according to the KEGG pathway classification. Moreover, the *desA* and *desB* genes, respectively encoding a $\Delta 5$ and a $\Delta 10$ desaturase, were also down-regulated at 12°C in the $\Delta casK/R$ strain compared to the WT strain. Finally, one gene (BC_1036) was putatively involved in glycerophospholipid biosynthesis. The *ykhA* gene (BC_5426) was up-regulated in the *casK/R* mutant and putatively belongs to the UFA biosynthesis pathway.

Nine genes (BC_1088, BC_3555, BC_4522, BC_4523, BC_4524, BC_4525, BC_4526, BC_5343, BC_5344) putatively encoding enzymes involved in FA degradation were down-regulated in both EP and SP. (Table 1).

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Table 1: Identification of the casK/R-regulated genes belonging to the pathways of fatty acid metabolism. Genes were identified as being casK/R-regulated with a significant difference (p-value threshold of 0.05) in expression between WT and $\Delta casK/R$ strains in early exponential (EP) and stationary phase (SP), respectively. The strains were grown at 12°C.

| Locus number | Putative function | metabolic pathway | Gene name | EP | | | SP | | |
|--------------|---|---|-----------|------|--------|----------|------|--------|----------|
| | | | | fold | Result | Pvalue | fold | Result | Pvalue |
| BC_1173 | 3-oxoacyl-[acyl-carrier-protein] synthase III | FA biosynthesis | fabH | 0.28 | down | 9.73E-06 | 0.38 | down | 4.88E-05 |
| BC_1174 | 3-oxoacyl-[acyl-carrier-protein] synthase | FA biosynthesis | fabF | 0.27 | down | 8.93E-06 | 0.43 | down | 4.66E-04 |
| BC_1216 | Enoyl-[acyl-carrier-protein] reductase | FA biosynthesis | fabI | 0.32 | down | 1.27E-04 | 0.54 | down | 4.41E-03 |
| BC_0400 | Delta10 fatty acid desaturase | Biosynthesis of unsaturated FA | desB | 0.15 | down | 5.12E-08 | 0.54 | down | 9.00E-03 |
| BC_2983 | Delta5 acyl-lipid desaturase, | Biosynthesis of unsaturated FA | desA | 0.21 | down | 6.23E-07 | 0.20 | down | 5.69E-09 |
| BC_5426 | Acyl-CoA hydrolase | Biosynthesis of unsaturated FA | ykhA | 2.84 | up | 4.59E-03 | 3.60 | up | 1.84E-03 |
| BC_1036 | Glycerol-3-phosphate dehydrogenase | glycerophospholip synthesis | glpD | 0.30 | down | 1.69E-05 | 0.37 | down | 4.83E-05 |
| BC_1088 | Long-chain-fatty-acid--CoA ligase | FA Degradation | lcfB | 0.38 | down | 1.61E-04 | 0.36 | down | 1.18E-05 |
| BC_3555 | Aldehyde dehydrogenase | FA Degradation/ glycerophospholip metabolism | dhaS | 0.33 | down | 4.92E-04 | 0.45 | down | 3.24E-03 |
| BC_4522 | | FA Degradation | etfB | 0.43 | down | 2.35E-03 | 0.39 | down | 5.16E-05 |
| BC_4523 | Electron transfer flavoprotein beta-subunit | FA Degradation | etfA | 0.34 | down | 7.94E-05 | 0.40 | down | 5.62E-05 |
| BC_4524 | 3-hydroxybutyryl-CoA dehydratase | FA Degradation | fadB | 0.27 | down | 1.00E-05 | 0.33 | down | 3.70E-06 |
| BC_4525 | Transcriptional regulator, TetR family | FA Degradation | fadR | 0.32 | down | 5.84E-05 | 0.41 | down | 1.31E-04 |
| BC_4526 | Long-chain-fatty-acid--CoA ligase | FA Degradation | lcfA | 0.29 | down | 2.15E-05 | 0.60 | down | 1.71E-02 |
| BC_5343 | 3-hydroxybutyryl-CoA dehydrogenase | FA Degradation | fadN | 0.54 | down | 5.98E-02 | 0.58 | down | 1.16E-02 |
| BC_5344 | Acetyl-CoA acetyltransferase | FA Degradation | fadA | 0.42 | down | 1.00E-03 | 0.55 | down | 5.81E-03 |

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Three major FAs are indicators of *B. cereus* low temperature adaptation

We analyzed the FA composition of both the $\Delta casK/R$ and WT strains during growth at low and optimal temperature when cells reached the stationary phase in order to understand the impact of the differential regulation of these 11 genes involved in FA metabolism in the mutant strain.

Distinct FA profiles were observed at 37°C compared to 12°C for both strains (Figure 2). The three FAs with the highest proportion were different when considering the growth temperature. For the WT strain grown at 12°C, majors FAs were i17:1(5), i15 and i13 FAs, while the major ones were i15, i16 and n16 FAs when grown at 37°C. Even if the i15 FA is the major FA at both temperatures, the proportion of this FA was almost 2 fold lower at 12°C compared to that at 37°C for both WT and $\Delta casK/R$ strains (Figure 2). The i17:1(5) FA proportion displayed the highest difference in the WT strain at 12°C compared to 37°C. This UFA was much less 12-fold less abundant at 37°C, with a proportion of just $1.45 \pm 0.32\%$ of total FA.

When comparing the WT and the $\Delta casK/R$ FA profiles, and more precisely the three major FAs, some difference could be noticed. The i17:1(5) UFA proportion accounted for $16.78 \pm 1.13\%$ of total FA in the WT strain vs $9.61 \pm 1.55\%$ of total FA in the $\Delta casK/R$ strain grown at 12°C. Considering the i13 FA, a significant difference was observed between the WT ($10.79 \pm 0.26\%$) and the $\Delta casK/R$ strain ($13.44 \pm 1.711\%$). For the i15 FA, no significant difference of proportion was observed between the two strains.

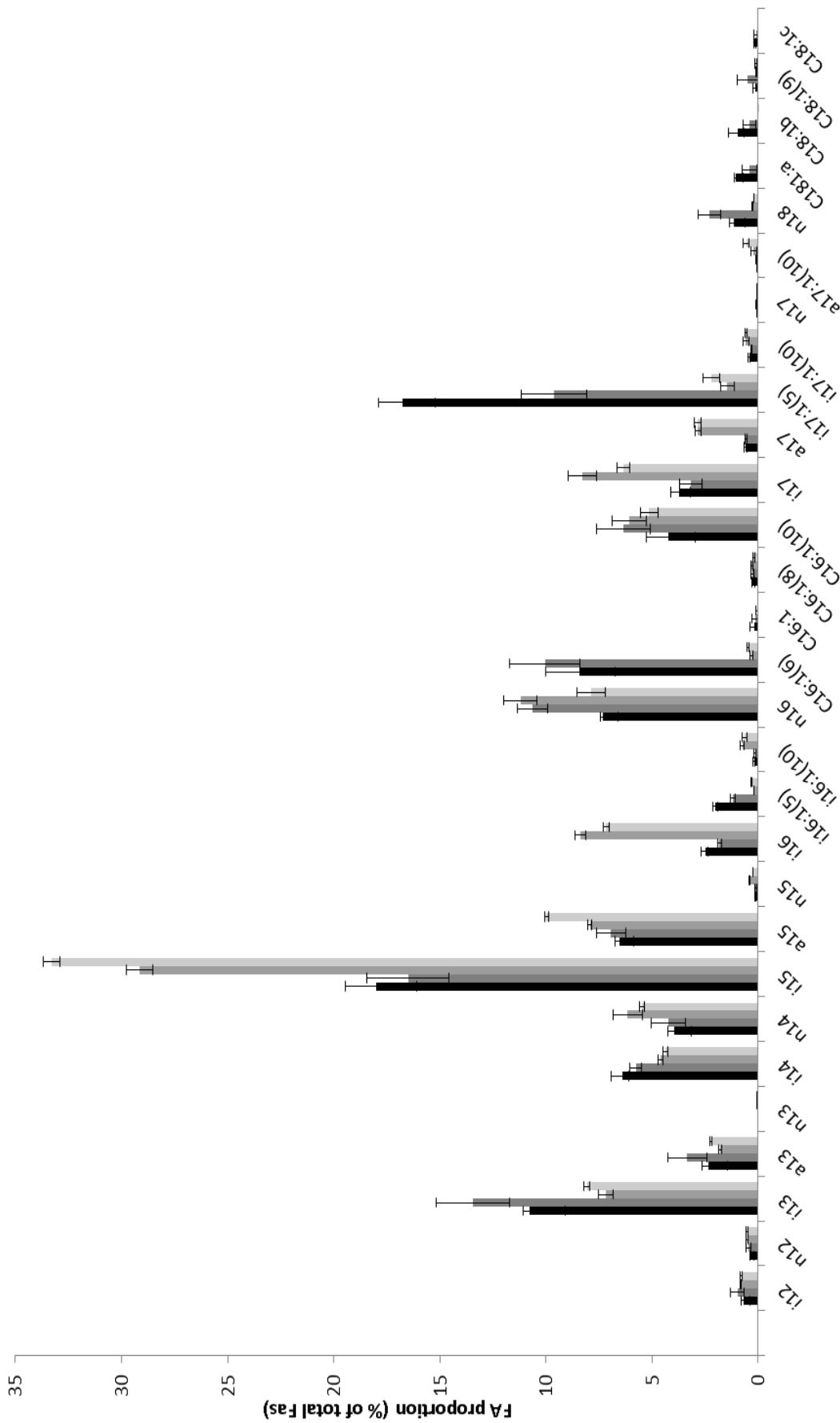


Figure 2: Figure representing WT and ΔcasK/R FA composition in percent of total fatty acids. The fatty acids were extracted in the stationary growth phase at 12°C (WT (■) and ΔcasK/R (■)) and 37°C (WT (■) and ΔcasK/R (■)). Each value is a mean of triplicate measurements with standard deviation calculated across triplicate measurements. FA nomenclature: iX = iso-FA with x number of carbons; aX = anteiso-FA with x number of carbons; nX = saturated FA with x number of carbons. When the FA was unsaturated, the symbol “:” prefixes the number of unsaturations into the FA chain.

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The $\Delta cas K/R$ strain displays a lower proportion of $\Delta 5$ -UFAs

The anteiso/iso ratio and the UFAs proportion were described to increase during *B. subtilis* growth at low temperature (Beranova et al., 2008) and were therefore checked here for both *B. cereus* strains.

The $\Delta casK/R$ strain displayed a slightly higher value of anteiso/iso ratio compared to the WT strain at 37°C as well as at 12°C (figure 3A). However, both strains, the ratio was slightly lower at low temperature indicating that this parameter was not indicative for *B. cereus* low temperature adaptation. In total, anteiso-branched-chain FAs represented 9.52 ± 0.44 % of total FAs at 12°C in the WT, while at 37°C this proportion was somewhat higher, 12.78 ± 0.08 % of total FAs.

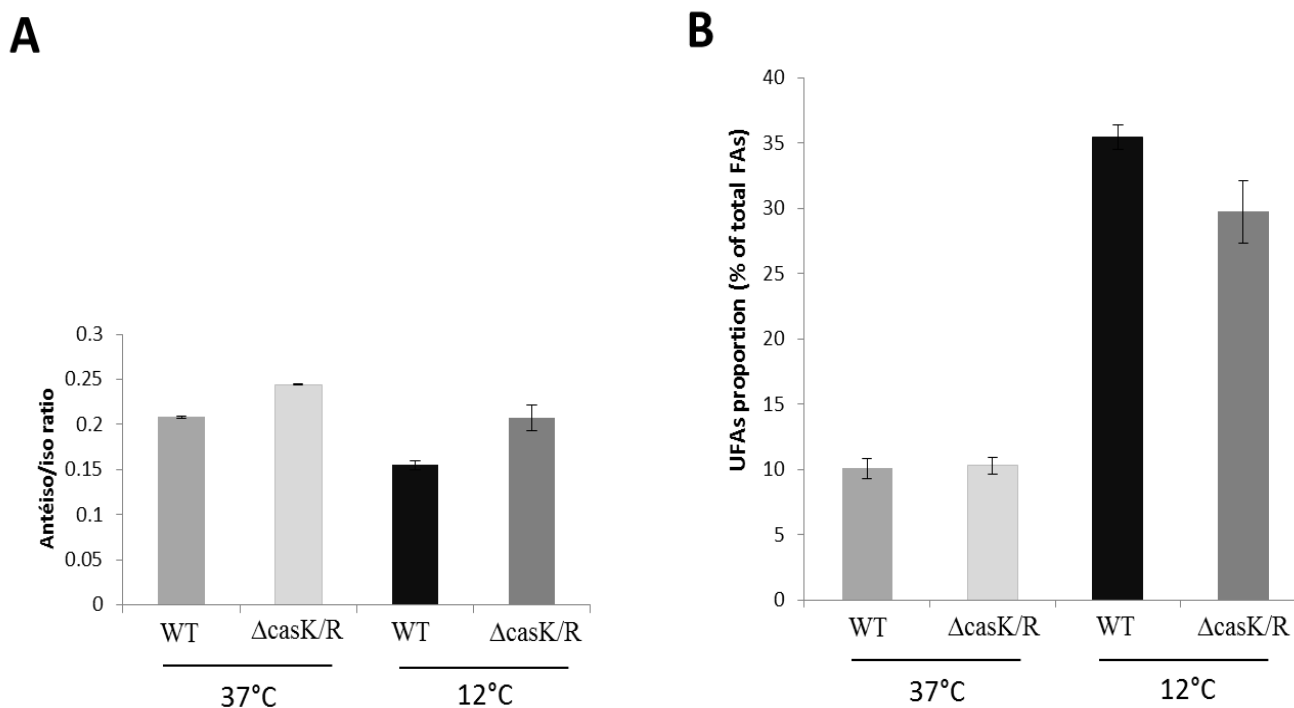


Figure 3: Anteiso/ iso ratio and UFAs proportion at 37°C (WT \blacksquare and \square $\Delta casK/R$) and 12°C (WT \blacksquare and \square $\Delta casK/R$). Each value is a mean of triplicate measurements with standard deviation calculated across triplicate measurements.

In both strains, the main change in FA membrane composition during low-temperature growth essentially is the proportion of UFAs (Figure 3B). It was significantly three-fold higher ($p < 0.05$, Student's t-test) at 12°C than at 37°C for both strains, and represented 35% of the total FAs for the WT. At 12°C, proportion of UFAs was slightly lower in $\Delta casK/R$ compared to the WT strain in three independent experiments, but differences were not significant

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($p > 0.05$, Student's t-test). However, when focusing on the proportion of FAs unsaturated at position $\Delta 5$, which are presumably unsaturated by the $\Delta 5$ -desaturase DesA, significant differences were observed between the two strains grown at 12°C ($p < 0.05$), with a higher proportion of $\Delta 5$ -UFAs observed in the WT strain ($18.80 \pm 1.09\%$ of total FA) compared to the $\Delta casK/R$ strain ($10.81 \pm 1.51\%$) (Figure 4A). The two strains grown at either 37°C or 12°C showed no significant differences for the UFAs on position $\Delta 10$, which are presumably desaturated by the $\Delta 10$ desaturase DesB, (Figure 4B).

Considering the remaining UFAs, unsaturated at position $\Delta 9$, $\Delta 8$ and $\Delta 6$, the type of unsaturation mechanism are not known. Whatever, no difference between the WT and the $\Delta casK/R$ strain was noticed (figure 4C).

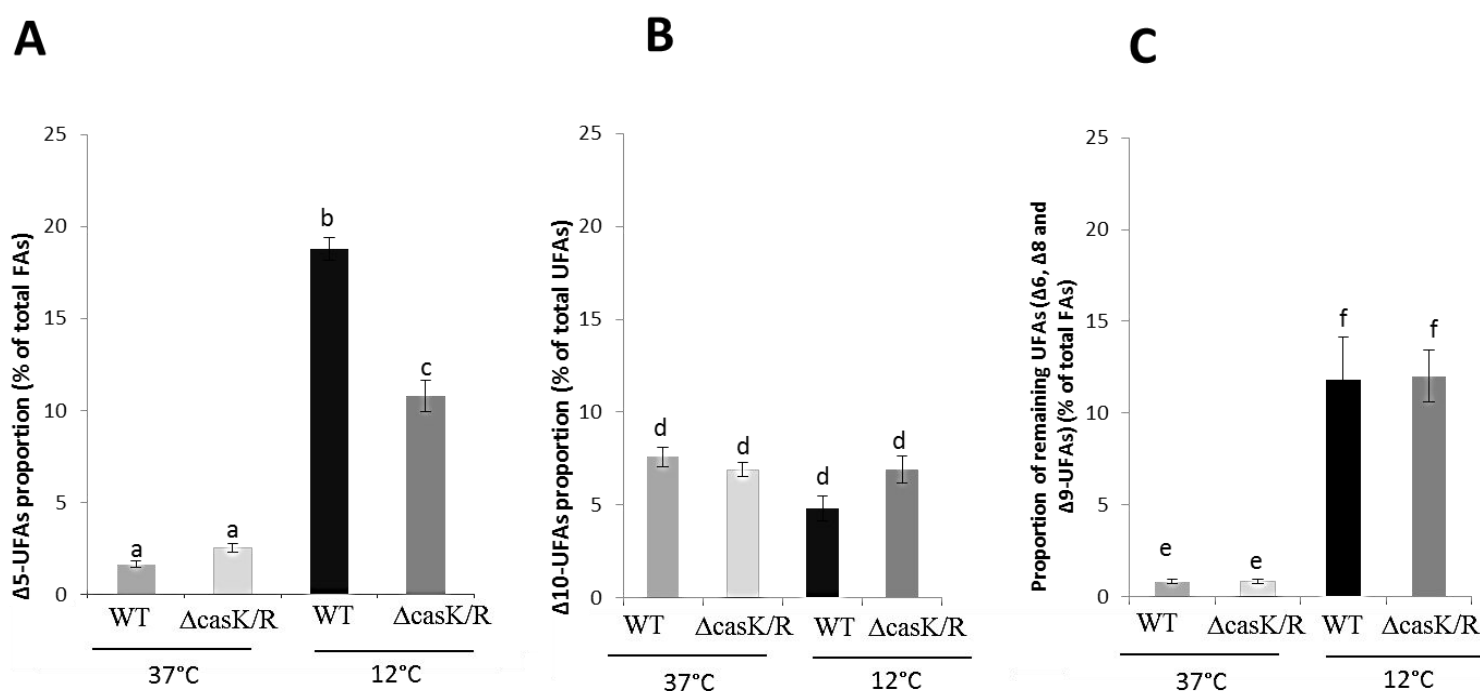


Figure 4 : $\Delta 5$ -UFAs proportion, $\Delta 10$ -UFAs proportion and remaining UFAs proportion at 37°C (WT \blacksquare and $\Delta casK/R$ \blacksquare) and 12°C (WT \blacksquare and $\Delta casK/R$ \blacksquare). The proportion of remaining UFAs was calculated as the $\Delta 5$ -UFAs and the $\Delta 10$ -UFAs proportions removed from the proportion of total UFAs. Each value is a mean of triplicate measurements with standard

Lecithin supplementation abolishes the cold-growth impairment of $\Delta casK/R$

The WT and the $\Delta casK/R$ strains were grown in a defined synthetic AOAC broth at 12°C in presence of various concentrations of purified lecithin (a UFAs source), without purified lecithin, or with hydrogenated-lecithin (lecithin in which all FAs had been saturated and containing only saturated FAs).

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When growth was performed at 12°C without lecithin, the $\Delta casK/R$ strain showed delayed growth and a lower final OD_{600nm} than the WT strain, indicating lower cold-growth adaptability (Figure 5A). Time to reach an OD_{600nm} of 0.15 was about 20 h longer for the $\Delta casK/R$, at 56.33±8.39 h, than for the WT strain, at 36.33±3.06 h.

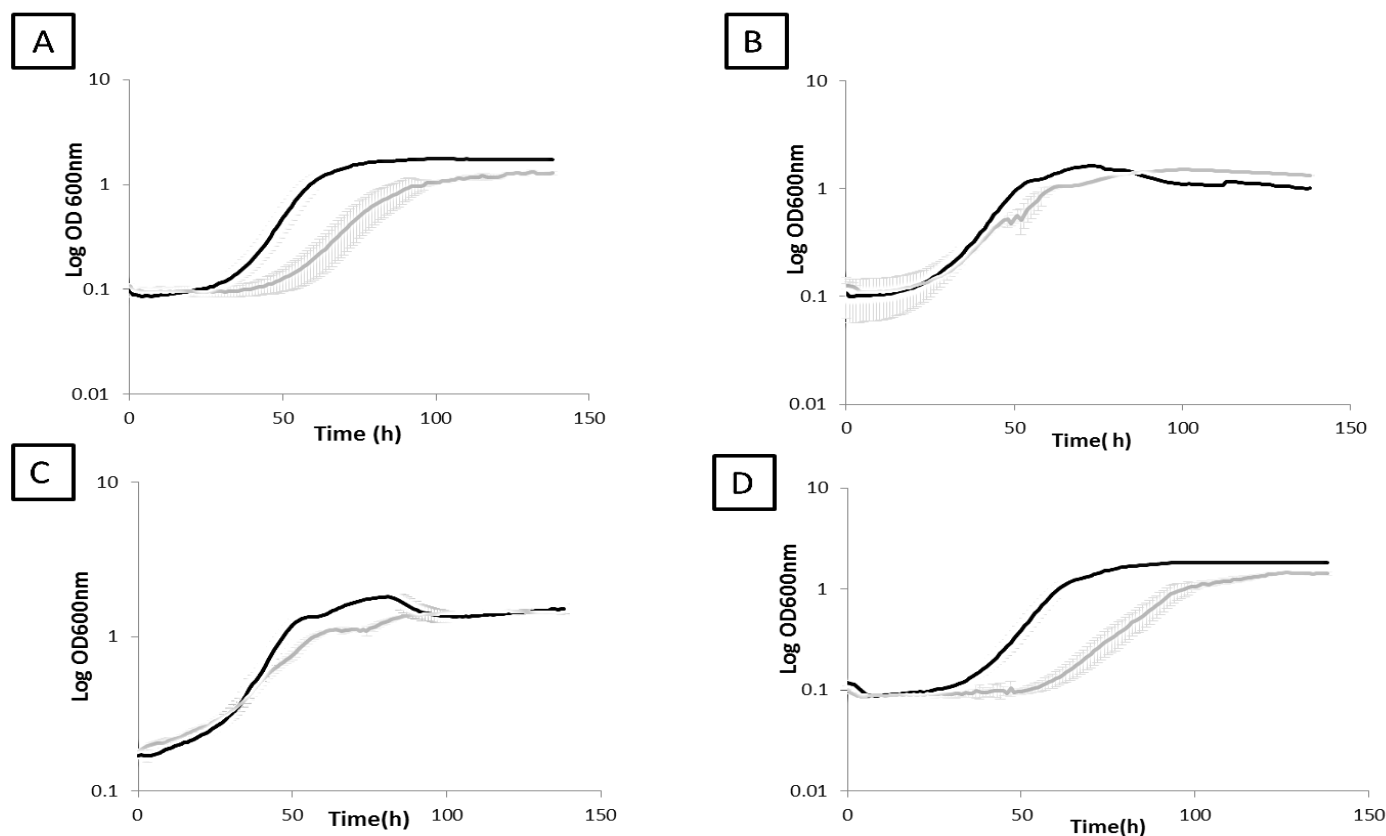


Figure 5: Growth curves of *B. cereus* ATCC 14579 WT (black) and $\Delta cas K/R$ (grey) strains at 12°C in an automatic turbidimeter. Strains were grown in AOAC medium (A), in AOAC containing lecithin at concentrations of 25 $\mu\text{g/mL}$ (B) and 375 $\mu\text{g/mL}$ (C), and in AOAC containing hydrogenated lecithin at a concentration of 375 $\mu\text{g/mL}$ (D). Each curve is the mean of three biological replicates, with grey vertical bars representing standard deviations.

However, in experiments carried out in AOAC medium containing 25 or 375 $\mu\text{g.mL}^{-1}$ of lecithin, there was no visible lag, and the two strains reached similar final OD values (Figure 5B and 5C). Supplementation with UFAs source improved the ability of the mutant to grow at low temperature, bringing it up to a similar growth level to the WT strain.

When growth was performed with hydrogenated lecithin, the saturated FA source (Figure 4-D), there was a 25.7 h delay differential between the strains, with the WT strain reaching an OD_{600nm} of 0.15 in 38 ± 1.73 h compared to 63.67 ± 4.73 h for the $\Delta casK/R$ strain. In this condition, supplementation with a saturated FA did not improve the mutant growth at low

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temperature.

These findings indicate that only supplementation with UFAs was able to abolish the cold growth delay of the $\Delta casK/R$ strain.

WT and $\Delta casK/R$ strains grown with lecithin displayed similar fatty acids profile

In order to determine whether the supplementation with lecithin changes membrane FA composition, we computed the FA profiles of WT and $\Delta casK/R$ strains grown at 12°C in AOAC-defined medium supplemented with or without lecithin.

Our results showed that the WT and $\Delta casK/R$ strains grown with lecithin displayed the same FA pattern (supplementary data Figure S1). This pattern was different from the pattern of the WT strain grown without supplementation. The major FA for the WT strain in AOAC medium without supplementation appeared to be the i17:1(5) like in LB medium. But when the medium was supplemented with lecithin, consequently rich in UFAs, the UFA C18:2 became the major UFA. Other UFAs presumably originating from lecithin incorporation were identified: C18:3 and C18:1. These two UFAs are not or poorly synthesized by *B. cereus*. This suggests that incorporation of UFAs from the lecithin change the FA profile of *B. cereus* strains.

Discussion

Several two-component systems (TCS) interacting with mechanisms of FA metabolism have been described in bacteria. For instance, PhoP/Q in *Salmonella enterica* plays a role in regulating outer membrane acidic glycerophospholipids in response to acidic pH and cAMP (Dalebroux et al., 2014). LasI/LasR and RhII/RhlR quorum sensing systems were shown to be involved in cell-cell communication in *Pseudomonas aeruginosa* by producing a fatty acid signal (Fernandez-Pinar et al., 2012). DesK/R was identified in *Bacillus subtilis* for its role in the maintenance of membrane fluidity, in response to a decrease in temperature, by regulating the expression of a $\Delta 5$ -desaturase encoded by the *des* gene (Aguilar et al., 2001a).

We previously identified CasK/R as a TCS playing a key role in *B. cereus sensu lato* cold adaptation. Here, we showed that, in *B. cereus* ATCC14579, the *casK/R* mutation caused the down-regulation of several genes involved in FA metabolism during cold adaptation. This observation is consistent with previous studies on *Bacillus* genus cold adaptation showing that

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a major change observed during cold acclimation was related to FA profile modifications (Mansilla et al., 2004, Russell, 1990, de Sarrau et al., 2012c, Brillard et al., 2010c, Haque and Russell, 2004b).

Our transcriptomic data showed that several genes involved in FA metabolism were differentially expressed in the $\Delta casK/R$ strain compared to the WT. Earlier studies in *B. subtilis* revealed that FabH, FabF and FabI catalyze to three different steps in saturated FA synthesis (Choi et al., 2000a, Schujman et al., 2008, Heath et al., 2000). FabH is involved in the first step of the condensation resulting in fatty acid elongation, while FabF plays an important role in the secondary elongation step, and FabI ends the FA biosynthesis (see supplementary data Figure S2). The genes encoding the two desaturases DesA and DesB were also down-regulated in the $\Delta casK/R$ strain compared to the WT strain. These desaturases are responsible for UFA biosynthesis in *B. cereus* ATCC14579 (Chazarreta Cifre et al., 2013). *ykhA*, which was up-regulated in the *casK/R* mutant, putatively belongs to the UFA biosynthesis pathway, as indicated in Kegg database (http://www.genome.jp/kegg-bin/show_pathway?bce01040+BC5426). It encodes a protein annotated as an acyl-CoA hydrolase presumably releasing coenzyme A from the UFA at the end of biosynthesis.

A search on the FA degradation pathway on the KEGG database (http://www.genome.jp/kegg-bin/show_pathway?bce00071) revealed that 8 of the 9 genes mentioned above belong putatively to different steps in the cycle of FA degradation into acetyl-CoA, and one gene putatively degrades the FA into aldehyde products. These data strongly suggest that CasK/R is involved in the regulation of FA metabolism by affecting key steps of this metabolic pathway.

Our transcriptomic data also showed that the *fadR* gene encoding the transcriptional factor FadR was down-regulated in the $\Delta casK/R$ mutant compared to the WT strain. FadR has a DNA binding activity which controls the expression of 10 genes primarily involved in FA degradation (Matsuoka et al., 2007, Fujihashi et al., 2014). Here, all the operon of FadR (BC_4522 to BC_4526). These data suggest that CasK/R may regulate the FA degradation via the *fadR* expression.

Regarding our results it clearly appeared that FA pattern of *B. cereus* WT strain changed in a temperature dependent manner. At low temperature as well as at optimal temperature, three major FAs were identified for each pattern. Two of them clearly appeared as biomarkers of growth temperature, the i17:1(5) UFA found in a higher proportion and the n16 in a lower proportion during low temperature growth. Our results also showed that proportion of UFAs

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was significantly higher in both WT and $\Delta casK/R$ strain during growth at 12°C compared to growth at 37°C, as previously described (Brillard et al., 2010c, Haque and Russell, 2004b). This was not the case for the ante-iso/ iso ratio, in agreement with previous studies on *B. cereus* (Brillard et al., 2010c, Haque and Russell, 2004b) suggesting that *B. cereus* recruits preferentially UFAs (about 24% at 12°C) than anteiso-branched-chain FA for cold adaptation, unlike *B. subtilis*. At both 37°C and 12°C anteiso-branched-chain FAs represented a low proportion of total FAs of *B. cereus* (about 10%). In this respect, *B. cereus* cold adaptation is different from *B. subtilis* cold adaptation in which the proportion of anteiso-branched-chain FA is higher (up to 60%) and the proportions UFAs lower (no more than 5 %) than in *B. cereus* at low temperature (Beranova et al., 2008, Weber et al., 2001).

The $\Delta casK/R$ and the WT strains display each a different FA pattern during growth at 12°C and more precisely different proportion in the i17:1(5) UFA. Basically, the proportion of $\Delta 5$ -UFAs, which are specifically unsaturated by DesA, was higher in the WT strain than in the $\Delta casK/R$ strain. *B. cereus* DesA is a $\Delta 5$ desaturase homologous to *B. subtilis* Des protein (Chazarreta Cifre et al., 2013) that catalyzes the fixation of double bonds in the $\Delta 5$ position on FA. An extensive investigation of the biosynthesis of UFAs in Bacilli (Fulco, 1969) showed that for some *Bacillus* species that synthesize $\Delta 5$ UFAs, such as *B. megaterium* and *B. subtilis*, proportion of $\Delta 5$ -UFAs increases when the bacteria are grown at low temperatures. This is consistent with our results showing a higher proportion of $\Delta 5$ -UFAs in both WT and $\Delta casK/R$ strains grown at 12°C than at 37°C.

The major UFAs displayed by *B. cereus* ATCC 14579 strain in its membrane lipids at 37°C were the $\Delta 10$ -UFAs, whereas at 12°C, *B. cereus* synthesizes both $\Delta 10$ and $\Delta 5$ isomers. The same observation was previously reported for another *B. cereus* strain (Kaneda, 1972) confirming that *B. cereus* strains possess a DesB-active $\Delta 10$ desaturase regardless of growth temperature, whereas a $\Delta 5$ desaturation system is induced at lower temperature.

Transcriptomic data showed that *desA* expression was reduced in the $\Delta casK/R$ strain compared to the WT during growth at low temperature. This observation may explain why the proportion of $\Delta 5$ -UFAs was lower in the $\Delta casK/R$ strain compared to the WT strain at 12°C, as shown whether in LB or AOAC media and may explain the $\Delta casK/R$ growth delay at low temperature (Diomandé et al., 2014).

A previous study showed that *B. cereus* cell membranes are able to incorporate exogenous UFAs (de Sarrau et al., 2013d) for optimal growth at low temperature. We therefore investigated the low-temperature growth of the $\Delta casK/R$ strain compared to the WT in a food

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grade UFAs source (i.e. lecithin)-supplemented medium. The same experiment was also performed with a saturated FAs (SFAs) source, i.e. hydrogenated lecithin. The results showed that only lecithin, a UFAs source, abolished the cold-growth impairment of the $\Delta casK/R$ mutant strain, and even when lecithin supplementation was as low as 25 $\mu\text{g/mL}$. However, the growth impairment was still observed when the medium was supplemented with hydrogenated lecithin, the SFA source, at a concentration as high as 375 $\mu\text{g/mL}$.

To enable bacterial membrane fluidification or metabolism, exogenous UFAs have to settle into the membrane or enter the cytoplasm, respectively (DiRusso and Black, 2004b). Studies on Gram-positive and Gram-negative bacteria have revealed that there are two types of transport for exogenous FA uptake: a passive type, also called simple diffusion, and an active type via a transporter, depending on FA chain length (Banchio and Gramajo, 1997). In *E. coli*, two proteins were shown to be responsible for the incorporation of exogenous FA into total lipids (Dirusso and Black, 2004a) : long-chain FAs (LCFAs) are proceeded by FadL and long-chain acyl-CoA by FadD (LCA-CoA).

There are two fates for exogenous FA: degradation by the β -oxidation pathway or incorporation in the membrane. Recent studies on *B. cereus* showed that the FAs originating from the uptake of exogenous phospholipids are incorporated in the membrane (de Sarrau et al., 2013d). Similarly, we found UFAs from lecithine incorporated in both WT and $\Delta casK/R$ mutant strain at 12°C. These exogenous UFAs compensated the FA membrane composition changes caused by the lack of CasK/R during *B. cereus* growth at low temperature, confirming that the $\Delta casK/R$ strain needs more UFAs to restore its growth ability at low temperature. As *B. cereus* is a foodborne pathogen, it is reasonable to hypothesize that the role of CasK/R may depend on the availability of UFAs in the environment or the food.

The majority of UFAs observed in the profiles of the strains grown with lecithin were UFAs with 18 carbons, most probably originating from the lecithin. Moreover, branched-chain FAs were also present in the membrane of the WT or $\Delta casKR$ grown with lecithin supplementation but absent in the blend of FAs from lecithin, suggesting that a *de novo* synthesis of FA occurred, this observation is in accordance with those made for other bacteria (Jackowski and Rock, 1986{von Wallbrunn, 2003 #30). All these data showed that even if *B. cereus* synthesizes a major specific type of UFAs for its growth at low temperature, this foodborne pathogen is also able to incorporate FA coming from the environmental medium including food-additive such as lecithine.

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DesK/R, a TCS involved in cold adaptation by controlling FA unsaturation, was previously described in *B. subtilis* (Mansilla et al., 2003). The *B. cereus* CasK/R and the *B. subtilis* DesK/R belong to the same family of TCS (Fabret et al., 1999b, Diomandé et al., 2014) but have their genes different genetic organizations. Indeed, both *desA* and *desB* are located on different loci from the *casK/R* locus in *B. cereus*, whereas in *B. subtilis* *desK/R* and the *des* gene are co-located on the same chromosomal region. Moreover, the two histidine kinases (HK) display no similarity within their N-terminal sensory domains. In addition, *in silico* analysis indicates that *B. cereus* CasK displays no transmembrane domain, which suggests that it belongs to those rare HK that have a cytoplasmic location. In contrast, a DesK membrane location in *B. subtilis* is critical for temperature-decrease sensing (Cybulski et al., 2010). All these differences suggest that *B. cereus* CasK/R and *B. subtilis* DesK/R share a different mechanism of cold sensing. In *B. subtilis*, DesK was shown to sense an increase in membrane thickness in response to a decrease in temperature, while DesR regulates the expression of the *des* gene encoding a desaturase responsible for maintaining membrane fluidity during growth at low temperature (Cybulski et al., 2010c, Cybulski et al., 2002).

According to Mansilla et al., 2003, UFAs and isoleucine also regulate the expression of the *des* gene in *B. subtilis* in a DesKR-dependent manner (Mansilla et al., 2003). Given the presumed cytoplasmic location of CasK in *B. cereus*, it is conceivable that the signal perceived cannot directly relate to a change in membrane thickness.

The data on the *B. cereus* CasK/R system reported here reveal that this TCS is involved in the regulation of UFA metabolism for cold adaptation.

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Chapitre 4:

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système à deux composants CasK/R
chez *Bacillus cereus***

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I- Introduction à l'étude

Les systèmes à deux composants (TCS) sont des mécanismes moléculaires bien décrits pour leur rôle dans l'adaptation à divers stress environnementaux chez les bactéries (Faralla et al., 2014, Chen et al., 2014, Dintner et al., 2014, Hiscox et al., 2014, Okkotsu et al., 2014)

B. cereus est une bactérie ubiquitaire, capable de s'adapter à un grand nombre de changement environnementaux (Diomandé et al., 2014, Abbas et al., 2014, de Sarrau et al., 2012c, Duport et al., 2006b, Pandiani et al., 2011b). Cette bactérie possède une cinquantaine de TCS (de Been et al., 2006b). Leur rôle dans l'adaptation de *B. cereus* adaptation est majoritairement méconnu.

Nous avons précédemment mis en évidence l'un de ces TCS, CasK/R pour son implication dans l'adaptation de *B. cereus* au froid (Diomandé et al., 2014). Ce TCS semble impliqué dans la régulation du métabolisme des acides gras et ainsi joue un rôle dans la modification de la composition en acides gras membranaires (Diomandé et al, soumis). En effet, l'absence de *casK/R* est responsable de l'augmentation réduite des acides gras insaturés en position $\Delta 5$, AG insaturés par la desaturase DesA et nécessaire pour une adaptation optimale de *B. cereus* à basse température (Diomandé et al, soumis).

Pour avoir plus d'informations sur le fonctionnement de CasK/R nous avons tenté de répondre à plusieurs questions :

- Comment est transcrit le locus de *casK/R* ?
- CasK/R régule-t-il sa propre expression?
- CasK/R régule-t-il l'expression du gène *desA* ?

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II- Stratégie envisagée

Tout d'abord, il paraissait utile de définir l'unité de transcription de *casK/R*, ceci a été possible par des expériences de RT-PCR ciblant les gènes du TCS ainsi que les gènes adjacents. Mais aussi de vérifier si le transcrit identifié ne changeait pas en fonction des conditions de températures testées.

Ensuite, une fusion transcriptionnelle du promoteur de *casK/R* avec le gène rapporteur *lacZ* a été réalisée, cette construction visait à transformer la souche ATCC 14579 WT et étudier la cinétique d'expression de *casK/R* à basse température et à température optimale comme indiqué précédemment (Bouillaut et al., 2005). Dans le but de comprendre l'impact de l'absence de *casK/R* sur l'expression de son promoteur, l'expérience décrite précédemment a été réalisée cette fois-ci chez la souche $\Delta casK/R$. La comparaison des deux cinétiques d'expression du promoteur (WT vs $\Delta casK/R$) nous a permis de déterminer le type de régulation que CasK/R exerce sur son promoteur. La même approche a été utilisée pour identifier le type de régulation qu'exerçait CasK/R sur les promoteurs du régulateur *rpiR* en aval de *casK/R* et sur celui de *desA*. Ensuite des expériences de retards sur gel ont permis de définir s'il s'agissait de régulation directe ou indirecte.

En parallèle, l'effet de la présence de sources d'AGI exogènes sur l'activité du promoteur de *desA* a été déterminé.

Pour une partie de cette étude, j'ai encadré une étudiante de DUT qui m'a aidé dans la réalisation de ces tâches.

III- Résultats-discussions

Les expériences de RT-PCR ont permis de mettre en évidence l'existence d'un gène codant pour un régulateur RpiR like au sein de l'opéron *casK/R* situé juste en aval des gènes *casK* et *casR*. Le dosage des activités promotrices a révélé la présence d'un promoteur pour l'opéron, assurant l'expression de *casK/R* et du gène *rpiR* mais aussi une transcription individuelle pour le gène codant pour le régulateur *rpiR*. Elles ont aussi permis de mettre en évidence que CasR réprimerait les deux promoteurs, celui de l'opéron *casK/R* et celui du gène *rpiR* à basse température. Bien que dans un tiers des cas un TCS régule l'expression de son propre

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promoteur, la régulation négative d'un TCS sur son propre promoteur est une situation originale mais pas décrite dans la littérature.

L'importance de réguler finement l'expression de *casK/R* a été mis en évidence par le fait qu'ajouter des copies supplémentaires de ce TCS chez la souche WT entraîne une perte d'adaptabilité de cette souche au froid, et ce de manière similaire au phénotype de la souche $\Delta casK/R$.

Par ailleurs, la répression qu'exerce CasK/R sur le promoteur du gène *rpiR* est observée quel que soit la température, y compris à 37°C, ce qui suggère que CasK/R joue un rôle chez *B. cereus* même à température optimale.

Avec le même type d'approche, nous avons aussi étudié l'impact de la mutation de *casK/R* sur l'expression du gène *desA*. Contrairement aux deux autres promoteurs testés, *desA* est positivement régulé par CasK/R. Cependant, il est quand même à noter que l'absence de CasK/R réduit mais n'abolit pas complètement l'expression de *desA* car chez le mutant au fur et à mesure de la croissance l'activité augmentait faiblement pour atteindre un pallier en phase stationnaire, même si cette valeur restait 4 fois plus faible que celle obtenue chez la souche WT. Cela suggère qu'en l'absence de CasK/R, d'autres mécanismes, des facteurs sigma ou d'autres régulateurs, peuvent activer l'expression de *desA* au froid mais de manière beaucoup moins importante que CasK/R.

L'expression de *desA* est cependant diminuée en présence de sources d'AGIs exogènes dans le milieu, à basse température. Ceci suggère que lorsqu'il y a suffisamment d'AGIs incorporables dans la membrane de *B. cereus* pour son adaptation au froid, il n'est pas nécessaire de mobiliser DesA pour les insaturations des AGs.

Les résultats d'interactions protéine/ADN obtenus in vitro suggèrent que la régulation exercée par CasR sur les promoteurs de *casK/R*, de *desA* et de *rpiR* n'est pas directe. Ceci suggère l'existence d'un intermédiaire de régulation c'est-à-dire un régulateur lui-même régulé par CasR avant de réguler les gènes nommés précédemment.

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Ces données ont permis de mieux comprendre le fonctionnement de CasK/R pour l'adaptation de *B. cereus* au froid même si certains aspects du mécanisme restent à élucider.

En effet, des questions demeurent : quel est le signal perçu par CasK au froid ? Quel(s) est (sont) les régulateur(s) intermédiaires régulant l'expression de *desA* et *casK/R* ? Le RpiR serait-il le régulateur intermédiaire à CasK/R pour la régulation de ces promoteurs ?

Cette éventualité n'a pas été négligée, d'ailleurs, des essais de purification de cette protéine ont été amorcés mais toutes les tentatives se sont soldées par des échecs.

Cette piste semblait d'autant plus intéressante que lorsque la souche mutante était complétée avec les trois gènes de l'opéron, la complémentation était non fonctionnelle, aucune restauration du phénotype n'étant observée (données non présentées). Ceci représente un argument en faveur d'un rôle important de RpiR pour contrôler ce phénotype adaptation au froid. Des études supplémentaires seraient nécessaires pour une réponse à ces questions.

Mieux définir l'unité de transcription de l'opéron *casK/R* pourrait aussi être intéressant pour parfaire la connaissance sur ce TCS. En particulier le +1 de transcription, qui pourrait être défini par des expériences de 5'RACE PCR.

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Expression and regulation of the gene encoding the CasK/R two component system in *Bacillus cereus*

Sara Esther Diomandé^{1,2}, Bénédicte Doublet^{1,2}, Florian Vasai^{1,2}, Véronique Broussolle^{1,2} and Julien Brillard^{1,2,3}.

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¹ INRA, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, 84000 Avignon, France

² Université d'Avignon, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, 84000 Avignon, France.

³UMR 1333 DGIMI, INRA-Université Montpellier, 34095 Montpellier cedex 5

Running head: Expression and regulation of *casK/R*

Corresponding author: julien.brillard@univ-montp2.fr

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Abstract

Two component systems (TCS) are described as crucial elements in bacteria environmental adaptation. CasK/R is a TCS which plays a role in the foodborne pathogen *B. cereus* cold adaptation and optimal unsaturation of fatty acids for cold adaptation. The current study highlights data about the expression and the regulation of *casK/R*. We showed that *casK/R* is co-transcribed with a gene encoding an RpiR-like regulator protein, forming thus the *casK/R* operon. Furthermore, additional copy of *casK/R*, in *trans*, in the WT strain caused a cold-growth impairment, similar to that observed for a *casK/R* mutant, suggesting that a fine regulation of this operon was necessary for the *B. cereus* cold adaptation. We also demonstrated that, the expression of *casK/R* operon was repressed at low temperature in stationary phase as an overexpression of the *casK/R* operon was observed in the *casK/R* mutant. A second promoter was identified upstream the *rpiR* gene, which was repressed in presence of CasK/R whatever the growth temperature. The *desA* gene, which encodes a FA desaturase, presumably responsible to provide UFAs for *B. cereus* low temperature adaptation, was down-regulated in the CasK/R mutant at low temperature.

This study provides insights on the mechanisms involving CasK/R in *B. cereus* at low temperature.

Keywords: TCS, cold adaptation, desaturase, RpiR, *B. cereus*

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Introduction

Two component systems (TCS) have extensively been described for their role in environmental stresses adaptation (Wang et al., 2013a, Bekker et al., 2006, Wick and Egli, 2004). These systems consist of two proteins partners, a histidine kinase (HK) and its cognate response regulator (RR). The HK senses the environmental change signal, is auto-phosphorylated and then transmits the information to the response regulator by a transfer of phosphate from an histidine residue of the HK to an aspartate residue of the RR (Stock et al., 2000). The RR then, binds to specific DNA regions in order to regulate the expression of genes for the environmental stress adaptation (Stock et al., 2000).

B. cereus is a Gram-positive, spore forming bacteria, representing one of the major causes of foodborne poisoning (Dierick et al., 2005a, Bennett et al., 2013b). This bacterium is ubiquitous and able to adapt to a wide range of environmental fluctuations (Stenfors Arnesen et al., 2008a). *B. cereus* comprises about fifty TCS (de Been et al., 2006b). The role of many of them in *B. cereus* adaptation is still unknown. Biological functions including the involvement in sporulation, biofilm formation, and host–microbe interactions were already predicted for some of these TCS (Abee et al., 2011a, Esbelin et al., 2009, Brillard et al., 2008b). TCS playing a role in cold adaptation were described in species such as *Clostridium botulinum* (Derman et al., 2013), *Bacillus subtilis* (Aguilar et al., 2001a), Cyanobacterium *Synechocystis* (Browse and Xin, 2001). In *B. cereus*, the CasK/R TCS was also shown to be involved in cold adaptation (Diomandé et al., 2014). The *B. cereus* cold adaptation involves the fatty acid (FA) metabolism (Haque and Russell, 2004b, Brillard et al., 2010c, de Sarrau et al., 2012c). It is mediated by CasK/R, which is required for an optimal increase of $\Delta 5$ -unsaturated FAs, FA unsaturated by the desaturase DesA, during low temperature adaptation (Diomandé et al, submitted). However, it remains unclear how CasK/R is regulated at low- and optimal growth temperature. To gain more insight in the functionalities of CasK/R, we investigated on the expression of CasK/R and its regulation at low- and optimal growth temperature.

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Materials and methods

Strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and *B. cereus* cells were routinely grown in Luria broth (LB) medium with vigorous agitation at 37°C or 12°C. As required, antibiotic concentrations used for bacterial selection were: erythromycin 5 µg mL⁻¹ or kanamycin at 150 µg mL⁻¹ for *B. cereus* and ampicillin 100 µg mL⁻¹ for *E. coli*. Bacteria with the Lac⁺ phenotype were identified on LB agar containing 50 µg mL⁻¹ X-Gal.

Nucleic acid manipulations

The extraction of Plasmid DNA from *B. cereus* and *E. coli* was performed by a standard alkaline lysis procedure using the Wizard SV miniprep purification system (Promega, Charbonnières, France) with an additional incubation step with 5 mg lysozyme to lyse *B. cereus* cells as previously described (Brillard and Lereclus, 2007a). Chromosomal DNA was extracted from *B. cereus* cells harvested in mid-log phase as described previously (Brillard and Lereclus, 2007a). Restriction enzymes and T4 DNA ligase were used as recommended by the manufacturer (Promega). Oligonucleotide primers were synthesized by Eurogentec (Seraing, Belgium). PCR was performed in a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer, Courtaboeuf, France) using Expand High-Fidelity DNA polymerase (Roche Applied Science, Meylan, France). Amplified DNA fragments were purified using a PCR purification kit (Roche) and separated on 0.7% agarose gels after digestion as previously described (Brillard and Lereclus, 2007a). Digested DNA fragments were extracted from agarose gels with a centrifugal filter device (Montage DNA gel extraction kit, Millipore, Molsheim, France). All constructions were confirmed by DNA sequencing (Millegen, Labege, France).

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Table 1: Strains and plasmids used in this work

| Strain or plasmid | Relevant genotype and characteristics ^a | Reference or source |
|--------------------------------|--|---|
| Strains | | |
| <i>B. cereus</i> ATCC 14579 WT | ATCC 14579, wild type, phylogenetic group IV | ATCC, USA |
| ATCC 14579 $\Delta casKR$ | WT ^{$\Delta bc2216-bc2217$} , Km ^r | (Diomandé et al., 2014) |
| <i>E. coli</i> TG1 | $\Delta(lac-proAB)$ supEthi hsd-5 (<i>F'</i> traD36 <i>proA</i> ⁺ <i>proB</i> ⁺ <i>lacI</i> ^q <i>lacZ</i> Δ M15), general purpose cloning host | Laboratory collection |
| <i>E. coli</i> ET12567 | <i>F</i> dam-13::Tn9 <i>dcm-6</i> <i>hsdM</i> <i>hsdR</i> <i>recF143</i> <i>zjj-202</i> : :Tn10 <i>galK2</i> <i>galT22</i> <i>ara14</i> <i>pacY1</i> <i>xyl-5</i> <i>leuB6</i> <i>thi-1</i> , for generation of unmethylated DNA | Laboratory collection |
| Plasmids | | |
| pHT304- <i>casK/R</i> | 2848 bp region surrounding <i>casK</i> and <i>casR</i> genes cloned in Sall and BamHI sites of pHT304-18 | (Diomandé et al., 2014) |
| pHT304- <i>casK/R</i> 'Z | 376 bp region upstream from <i>casK/R</i> start codon inserted between <i>Hind</i> III and <i>Bam</i> HI sites of pHT304-18'Z | This study |
| pHT304- <i>rpiR</i> 'Z | 329 bp region upstream from <i>rpiR</i> start codon inserted between <i>Hind</i> III and <i>Bam</i> HI sites of pHT304-18'Z | This study |
| pHT304- <i>desA</i> 'Z | 634 bp region upstream from <i>desA</i> start codon inserted between <i>Hind</i> III and <i>Bam</i> HI sites of pHT304-18'Z | This study |
| pHT304-18'Z | Ap ^R and Em ^R cloning vehicle; <i>lacZ</i> reporter gene | Laboratory collection |
| pET101/D-TOPO | Ap ^R cloning and expression vehicle | Champion™ pET Directional TOPO® Expression Kits |

^aKm, kanamycin; Ap, ampicillin; Em, erythromycin

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RT-PCR experiments

Total RNA was extracted from *B. cereus* ATCC 14579 WT and $\Delta casK/R$ cells grown aerobically in LB medium at OD₆₀₀ = 1.0 at 37°C and at 12°C. A mechanical cell disruption was performed by adding 0.3 g sterile 0.1-mm silica beads (MP Biomedicals) followed by one run of 45 sec at speed 6 on a FastPrep Instrument (FP120). cDNA synthesis from 1 µg of total RNA was performed by using AMV-RT polymerase according to the instructions given by the RT-PCR kit (Roche). Specific amplifications were performed with Expand-High Fidelity polymerase as recommended (Roche), using the primers RT-BC2218+_fw and RT-BC2218+_rv (Table2) for the BC_2218 gene, RT-BC2216-17_fw and RT-BC2216-17_rv for the *casK/R* genes.

Table 2: Primers used in this study.

| Oligonucleotides | Sequence (5' - 3') ^a |
|------------------|---|
| RT-BC2217-18_fw | CGAAAAGAGAGCAAGAGGTA |
| RT-BC2217-18_rv | ATATGTTTCGGCAATACTTCG |
| RT-BC2216-17_fw | AACAAGTAAGGGAATCAGCA |
| RT-BC2216-17_rv | ATTGATAACCATCTGCTCCA |
| RT-BC2218+_fw | GTACAAGAAGTGGGATTGGA |
| RT-BC2218+_rv | TAATACCAGAGCCACCATTT |
| RT-BC2218+ | CGAAAAGAGAGCAAGAGGTA/CGAAGTATTGCCGAACATAT |
| P_D5des-L-Hind | CGGCAAGCTTAGGTGAAGGGTTCTTAGGAG |
| P_D5des-R-Bam | CGTCGGATCCATTTCCTTTTGTGTTGC |
| P-BC2216-Fw-Hind | TGGCAAGCTTCGTCGGTGTACAGACTTAAA |
| P-BC2216-Rv-Bam | TGGCGGATCCCAACTACAATTATAACATTAAGAA |
| P-BC2218-Fw-Hind | TGGCAAGCTTAGATTGCAGCAGAACTTCATA |
| P-BC2218-Rv-Bam | TGGCGGATCCCCTTCCATCGTAGCTTTA |
| EMSA-P-BC2216 F | CGTCGGTGTACAGACTTAAA |
| EMSA-P-BC2216-R | AACTACAATTATAACATTAAGAA |
| EMSA-P-BC2218-F | AGATTGCAGCAGAACTTCATA |
| EMSA-P-BC2218-R | TCCCCTTCCATCGTAGCTTTA |
| P_D5des-F-EMSA | AGTTACCGAAACTAGGACTG |
| P_D5des-R-EMSA | ATTCCTTTCCTTTTGTGTTGC |
| ssubioF | GGTAGTCCACGCCGTAAACG |
| ssuR | GACAACCATGCACCACCTG |

^a Restriction enzyme sites are underlined

The primers RT-BC2217-18_fw and RT-BC2217-18_rv were used for the region overlapping *casR* and BC_2218. The primers RT-BC2216-17_fw and RT-BC2217-18_rv were used for a region overlapping *casK*, *casR* and BC_2218.

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Construction of *lacZ* transcriptional fusions and β -Galactosidase assay

The DNA fragment harboring the *desA*, *rpiR* and *casK/R* promoter region was PCR-amplified using the primers listed in Table 2. The amplified DNA fragment was then digested according to the endonuclease sites introduced in the primers. *lacZ* transcriptional fusion was constructed by cloning this DNA fragment between the corresponding sites of the low-copy plasmid pHT304-18'Z (Agaïsse and Lereclus, 1994). The recombinant plasmid obtained in each case was introduced into *B. cereus* ATCC 14579 WT and $\Delta casK/R$ strains by electroporation. *B. cereus* strains harboring this plasmid were cultured in LB medium at 12°C or 37°C. Cells were harvested all along the growth.

Cell disruption was performed by adding 0.3 g sterile 0.1-mm silica beads (MP Biomedicals) followed by one run of 45 sec at speed 6 on a FastPrep Instrument (FP120). β -galactosidase specific activities were measured in triplicate samples from each culture as described before (Bouillaut et al., 2005) and expressed in units of β -galactosidase per mg protein (Miller units). Total proteins in the sample were quantified using the Bradford assay (BioRad protein assay). Specific activity experiments were repeated three times. Error bars indicate standard deviations of triplicate measurements.

Supplementation experiments with exogenous source of UFAs

B. cereus ATCC 14579 WT and $\Delta casK/R$ strains containing the pHT304-*desA*'Z (Table 1) were cultured at 37°C until reaching an OD₆₀₀ value of 0,35. Then the cultures were divided in two, one supplemented with purified soja lecithin, an unsaturated fatty acid source and the other non-supplemented and incubated at 12°C for 24h. The OD₆₀₀ was measured and no culture growth was noticed indicating that the cells were in a lag phase at low temperature. The WT and $\Delta casK/R$ cells were then harvested and a β -Galactosidase assay was performed in order to determine the *desA* promoter activity in these conditions.

Purification of the CasR protein

The *casR* ORF was PCR amplified from *B. cereus* ATCC 14579 DNA using the oligonucleotides, pET-RR2217*Fw* and pET-RR2217*Rv*. The amplicon was introduced into pET101/D-TOPO (Invitrogen) by TA cloning. The inserted sequence was confirmed by DNA sequencing. The resulting construct was transformed into *E. coli* BL21-CodonPlus(DE3)-RIL strain (Stratagene) for protein production. BL21-CodonPlus(DE3)-RIL cells carrying the pET101-*casR* expression plasmid were grown in 4 L LB medium containing 100 $\mu\text{g.mL}^{-1}$

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ampicillin, at 37°C with agitation (200 rpm) until the cell density reached an OD₆₀₀ of about 0.5. Then, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture, and growth was continued for two hours. Cells were harvested by centrifugation (6500 rpm for 10 min at 4°C), and the pellet was washed, and resuspended in 40 mL ice-cold extraction buffer consisting of 1mM of phenyl-methane sulfonyl fluoride (PMSF), phosphate buffer saline (PBS) 1X, and a protease inhibitor cocktail (one tablet of Complete Mini, EDTA free, Roche). Cells were then incubated with 0.5 mg/mL lysozyme for 30 min under gentle agitation at 4°C and disrupted by sonication for 15 min at 4°C using a transonic 780h sonicator. The lysates were clarified by centrifugation at 8000 rpm for 30 min at 4°C and loaded onto a 4 mL Ni²⁺-immobilized metal affinity chromatography column (HisPur Ni-NTA Resin from ThermoScientific), equilibrated with the extraction buffer. The column was washed with 20 mL of extraction buffer containing 20 mM imidazole in PBS 1X. Then CasR was eluted with 2 mL extraction buffer containing 250 mM imidazole in PBS 1X. The eluted fraction was desalted by dialysis, concentrated using Microcon 10 kDa-molecular-mass cutoff devices (Millipore), and stored at -20°C in 10% glycerol until analysis. The purity of the protein was estimated to be above 90% by Coomassie blue-stained SDS-PAGE. The protein concentration was determined using a Bradford assay (Interchim) with bovine serum albumin as the reference.

CasR *in vitro* phosphorylation assays.

First, the His-tag used for the CasR purification was cleaved by the EnterokinaseMax™ (EKMax™) kit as recommended (Invitrogen). The reaction comprised 400 µl of CasR at 1.5mg/ml (corresponding to 600 µg of CasR protein) resuspended in a mix containing 90µl of 10X EKMax™ Buffer, 120 µl of EKMax™ and 290 µl of water. The reaction mix was then incubated at 37°C overnight (~16 hours).

Then the CasR phosphorylation reaction was performed in binding buffer containing 20 mM HEPES (pH 7.9, Sigma- Aldrich), 60 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol (Invitrogen), 0.3 mg/ml BSA, 5 % glycerol and 50 mM lithium potassium acetyl phosphate (Sigma-Aldrich) for 1h at room temperature as previously described (Dahlsten et al., 2014a).

The phosphorylation state of the resulting product was tested by using the Phos-tag™ Acrylamide from the Wako pure chemical industries. The phosphorylated His-tag cleaved CasR was first concentrated with the Centricon kit from Millipore. Then 2.5 µg of protein

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(3 μ l) were loaded on the Phos-tagTM gel. Non-phosphorylated but His-tag-cleaved CasR protein and the phosphorylated but non-his-tag-cleaved CasR were used as controls. After running at 120V, for 2h, the gel was Coomassie blue-stained.

Electrophoretic mobility shift assays (EMSA)

The nucleic acid fragments harboring the promoter regions of *casK/R*, *rpiR* or *desA* were PCR-amplified using the following biotinylated forward primers, respectively EMSA-P-BC2216 F, EMSA-P-BC2218-F and P_D5des-F-EMSA and non-biotinylated reverse primers, respectively EMSA-P-BC2216-R, EMSA-P-BC2218-R and P_D5des-R-EMSA (Table 2). A DNA fragment of ssuRNA BC0007 sequence ([NC_004722](#)), which was amplified with the biotinylated ssuBioF and ssuR primer pair, was used as negative control DNA. The 5'-labeled amplicons were purified using the High Pure PCR Product Purification Kit (Roche). The binding reactions were performed for 30 min at 37°C by incubating 2nM of biotin-labeled DNA fragments with different amounts of CasR in 10 mM Tris-HCl buffered at pH 7.5, and containing 50 mM KCl, 2.5% glycerol, 5 mM MgCl₂ and 5 mg/L poly(dI-dC). The samples were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel and electrotransferred onto Hybond N+ Nylon membranes (Amersham) using the Invitrogen iBlot Cell Transfer System. Biotin-labeled DNA was detected using the LightShift Chemiluminescent EMSA Kit (Pierce).

Statistical analysis

All data were analyzed using the student's t-test. The null hypothesis H₀ was rejected at $p(H_0) < 0.05$ or $P(H_0) < 0.001$ depending on the experiment.

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Results

Transcriptional units of the *casK/R* locus

RT-PCR experiments were carried out on RNAs extracted from cells harvested at the entry into stationary at 37°C and 12°C. Four couples of primers were used (Figure 1). The RT-PCR results revealed the detection of mRNA overlapping the *casK* and *casR* genes and also the *casR* and *rpiR* genes (Figure 1). These results showed that *casK/R* are co-transcribed with a RpiR-like regulator encoding gene and thus that *casK*, *casR* and the RpiR-like encoding gene constituted an operon.

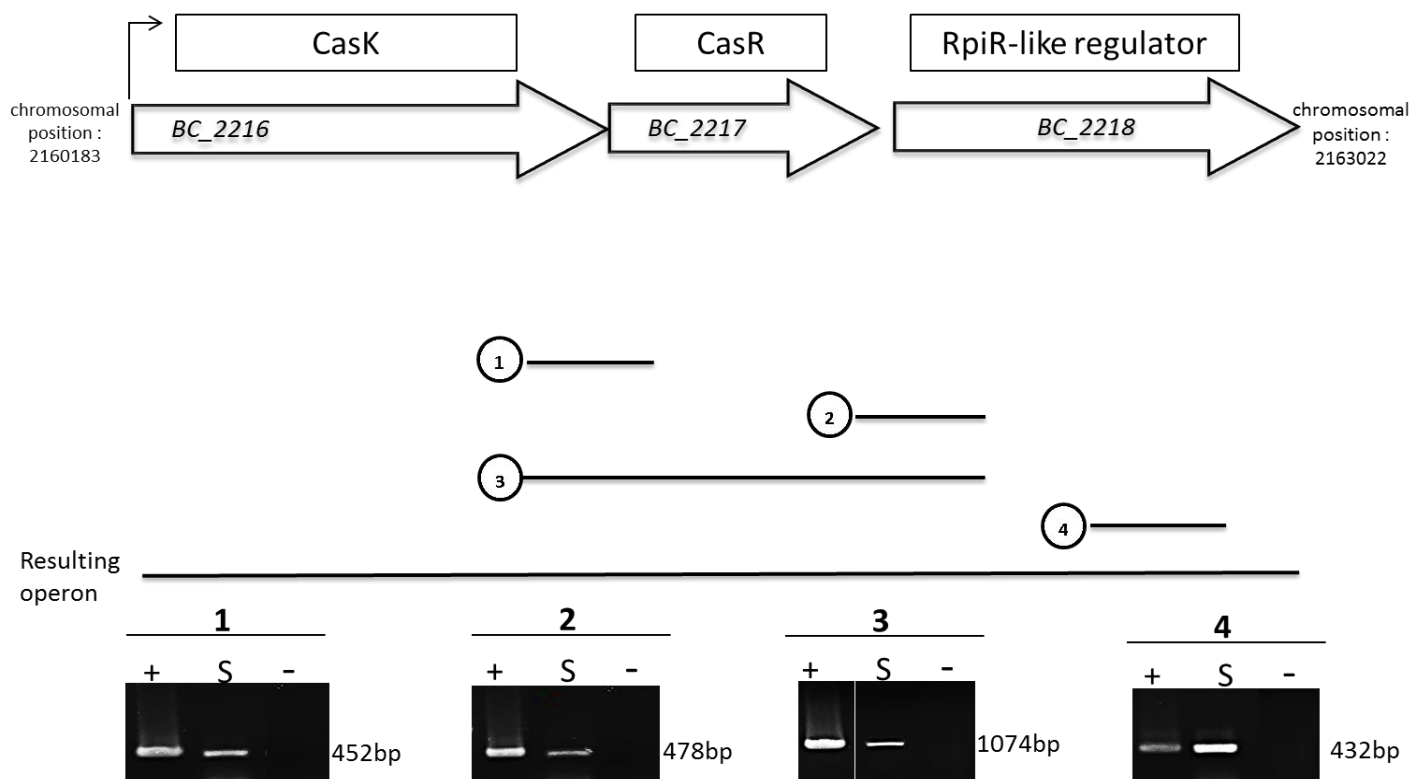


Figure 1: RT-PCR detection of the *casK/R* operon in *B. cereus* strain ATCC 14579. On the top of the figure, the CasK, casR and *rpiR* locus are represented, just below are identified the regions targeting by primers for the experiments, these regions are numbered from 1 to 4. RT-PCR results are at the bottom of the figure, Lane “+” represents the positive control (PCR on genomic DNA). Lanes “S” represents the RT-PCR on 500 ng RNA. Lanes “-” represents negative control (RT-PCR without RNAs). Numbers refer to the positions of the RT-PCR products on the *casK/R* locus. RNA extraction was performed on strains grown at 37°C in LB broth and harvested in stationary phase ($OD_{600}=1$). Whatever the temperature tested the RT-PCR results obtained were the same and this figure describes the results obtained at 37°C.

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Expression of *casK/R* at low- and optimal growth temperature

We studied the expression of the *casK/R* promoter in both *B. cereus* WT and $\Delta casK/R$, using a transcriptional fusion between the *casK/R promoter* region and the *lacZ* reporter gene. *B. cereus* WT and $\Delta casK/R$ strains carrying the plasmids pHT304-*casK/R*'Z (Table 1) were grown in LB medium at 12°C and 37°C, and β -galactosidase activity was quantified all along the kinetics of growth (Figure 2). In order to compare the *casK/R* promoter activity in both strains, despite the growth delay of the $\Delta casK/R$ strain at low temperature, kinetics of growth were expressed relatively to the entry in stationary phase, defined as time 0 on the x-axes (Figure 2A and 2B).

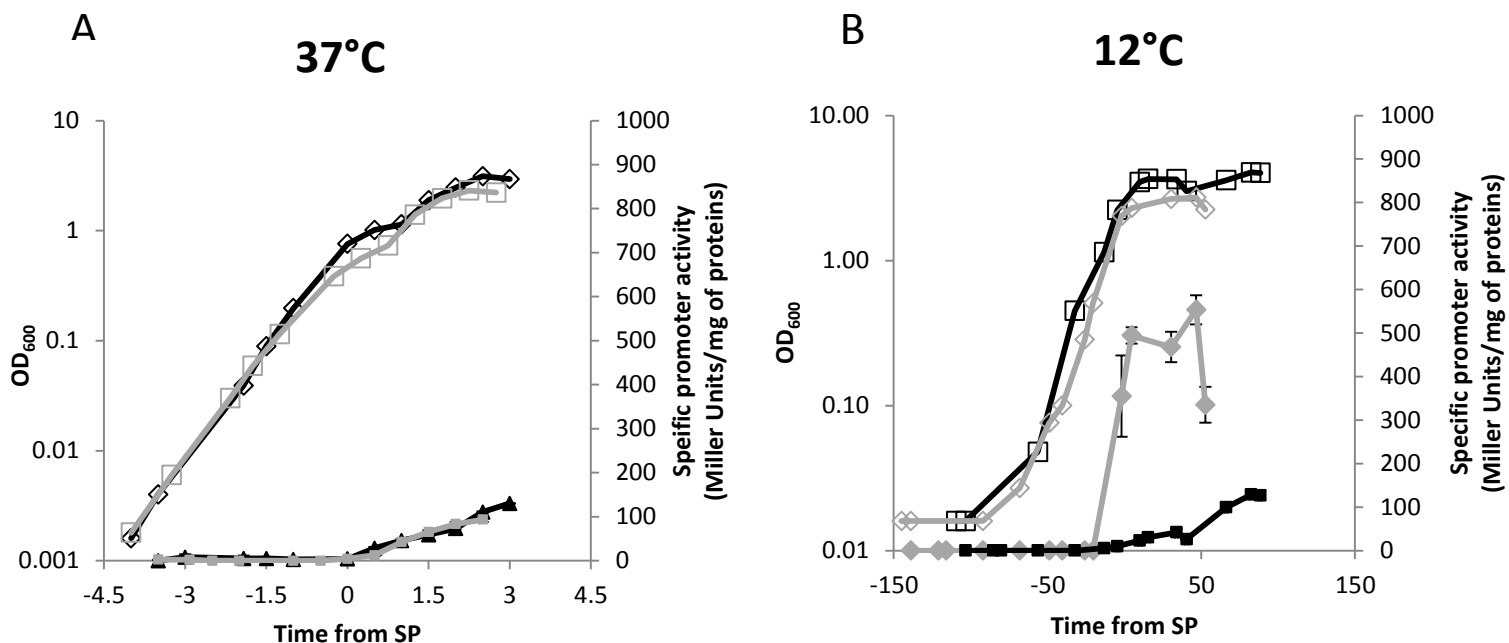


Figure 2: *CasK/R* directed *lacZ* expression in *B. cereus* WT or $\Delta casK/R$ mutant strains. Growth curves of the WT (black with empty symbols) and the $\Delta casK/R$ (grey with empty symbols) strains are represented at 12°C (A) and 37°C (B). β -galactosidase activity was measured in either the WT strain (black with full symbols) or in the $\Delta casK/R$ mutant (grey with empty symbols) harboring pHT304-*casK/R*'Z, during growth at 12°C (A) and 37°C (B) in LB medium. Time 0 indicates the onset of stationary phase

At 37°C, the *casK/R* promoter displayed an increase of activity after the entry into stationary phase (SP) in both WT and $\Delta casK/R$ strain (Figure 2A). But, whatever the growth phase, no significant difference was observed between the two strains.

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At 12°C, the *casK/R* promoter activity started increasing in the WT strain, at the entry into stationary growth phase (Figure 2A). But in the $\Delta casK/R$ strain, this overexpression is observed slightly earlier (about 20h before the entry into stationary phase) (figure 2A).

Our results indicate that at 12°C, at SP+30h the *casK/R* promoter activity was significantly higher in the $\Delta casK/R$ strain (487.67 ± 9.94 Miller Units/ mg of proteins) than in the WT strain (41.67 ± 15.90 Miller Units/ mg of proteins), a 10 fold difference was observed. This overexpression of *casK/R* promoter in the mutant strain in which *casK/R* is absent suggest that in the WT strain grown at low temperature, CasK/R negatively regulates its own promoter activity.

The addition of a *casK/R* copy in *trans* in the WT strain leads to a special phenotype at low temperature

Introduction of an additional *casKR* copy in *trans* in the WT strain was performed. The growth ability at low temperature of the recombinant strain was investigated. Growth of the ATCC 14579 WT and $\Delta casK/R$ strains, with or without complementation with the plasmid containing the *casK/R* region (pHT*casK/R*), were performed in LB medium at 12°C. As previously described, the $\Delta casK/R$ displayed growth impairment at low temperature when compared to the WT strain (Figure 3), and the complementation of the $\Delta casK/R$ strain with the pHT*casK/R* restored the cold growth ability.

Interestingly, the recombinant WT strain (harbouring pHT*casK/R*), displayed a cold-growth impairment, similar as that observed for the $\Delta casK/R$ mutant strain. As a control, growth of the $\Delta casK/R$ and the WT strain complemented with an empty plasmid i.e. pHT plasmid without

casK/R, were also performed at low temperature in LB medium. No difference of growth was observed between these transformed strains and the $\Delta casK/R$ and the WT strains, respectively (data not shown).

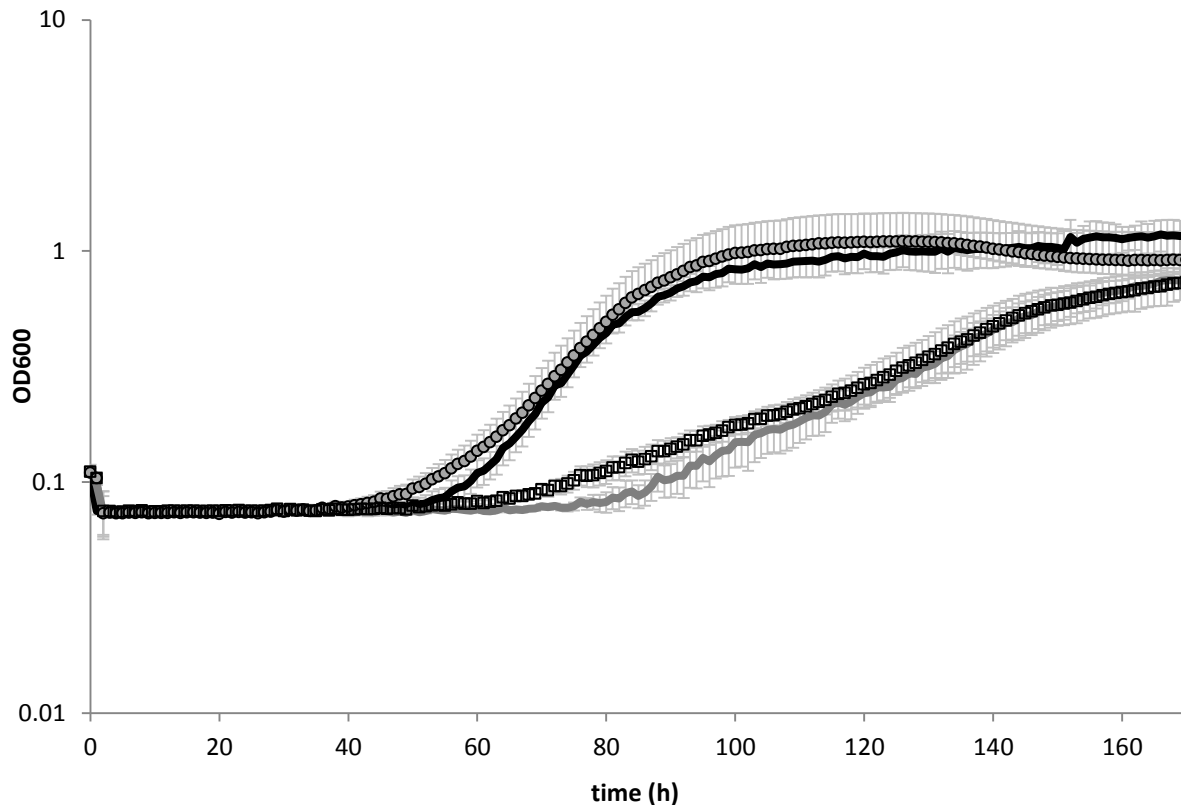


Figure 3: Growth of the ATCC 14579 WT strain (black line), $\Delta casK/R$ strain (grey line), WT+Pht304-casK/R strain (empty square), $\Delta casK/R$ +pHT304-casK/R strain (grey circle) at 12°C in an automatic turbidimeter.

This indicates that the growth impairment (for the WT recombinant strain) or the restoration of growth (for the mutant complemented strain) observed at low temperature was not due to the presence of the plasmid alone, but was due to the *casK/R* copy introduced in *trans* in these strains.

The *rpiR* gene has its own promoter

The *rpiR* gene belongs to the *casK/R* operon, which is regulated by CasK/R. However, RT-PCR experiments were performed in the mutant strain, to determine if *rpiR* could still be transcribed in the *casK/R* mutant (Figure 4). Primers targeting the *rpiR* transcript were used on RNAs extracted at different growth phase: start of the exponential phase (ESP), end of the exponential phase (EEP) and stationary phase (SP). When in the WT strain the presence of a transcript is expected, *rpiR* transcripts were also observed in the $\Delta casK/R$ strain, in which the *casK/R* genes are truncated, whatever the growth phase studied. This strongly suggested the existence of second promoter region for the *rpiR* gene in the 204 pb *casR-rpiR* intergenic region.

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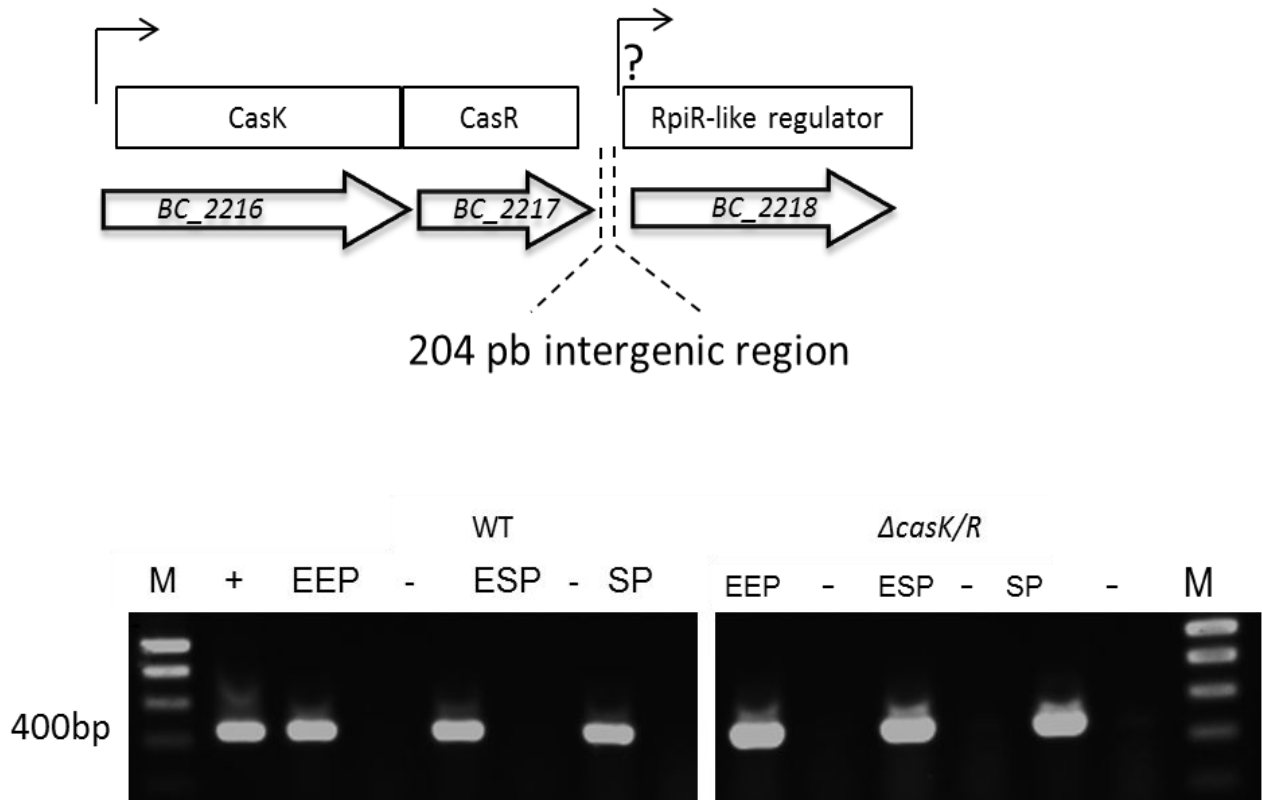


Figure 4 : RT-PCR detection of the *RpiR* gene in *B. cereus* ATCC 14579 WT and $\Delta CasK/R$ strains. On the top of the figure, the *casK*, *casR* and *rpiR* locus are represented. RT-PCR results are at the bottom of the figure, for the WT (left) and $\Delta CasK/R$ (right) strains. Lane “+”: represents the positive control (PCR on genomic DNA). Lanes “ESP”, “EEP” and “SP” respectively represents the RT-PCR on 500 ng RNAs harvested at the entry into exponential phase, the entry into stationary phase and in stationary phase. Lanes “-“ represents negative control (RT-PCR without RNAs). Lanes “M” represents the molecular marker profile. RNA extraction was performed on strains grown at 37°C in LB broth.

The individual promoter of the *rpiR* gene is under the control of CasK/R

The existence of a putative *rpiR* promoter in the *casR-rpiR* intergenic region was investigated. For this purpose, a transcriptional fusion between the putative promoter region and the *lacZ* reporter gene was constructed. *B. cereus* WT and $\Delta casK/R$ strains carrying the plasmids pHT304-*rpiR**lacZ* were grown in LB medium at low temperature or optimal growth temperature. A β -galactosidase activity was detected whatever the condition tested confirming that the existence of a promoter allowing the expression of the *rpiR* gene (Figure 5A and 5B). The *rpiR* promoter activity increased regularly during the exponential growth phase, for both strains grown at both tested temperatures. It was detected above the detection threshold when

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cultures reached an OD of about 0.1 units, which approximately corresponded to 35h during growth at 12°C (Figure 5A) and 2.5h at 37°C (Figure 5B) before the entry into stationary phase for both strains studied.

In the $\Delta casK/R$ strain, the *rpiR* promoter activity was significantly higher ($p < 0.05$) than in the WT strain, whatever the temperature tested. Indeed, the promoter activity in the mutant was about 2 fold higher in this strain when compared to the WT strain, at 12°C (respectively 70.00 ± 15.27 vs 32.66 ± 1.22 Miller units/mg of proteins) and at 37°C (respectively 118 ± 4.89 vs 55.00 ± 7.35 Miller units/mg of proteins). This suggests that this *rpiR* promoter is negatively regulated by CasK/R.

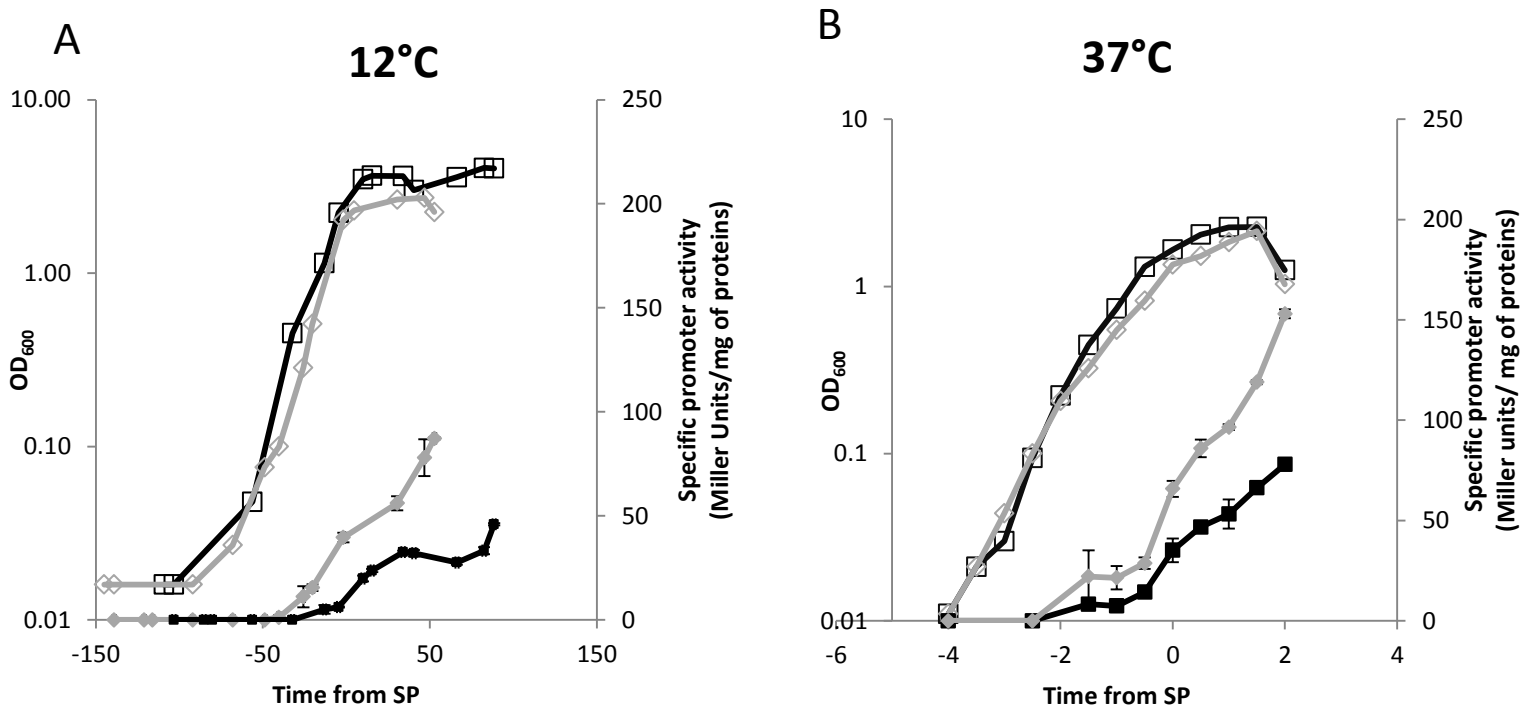


Figure 5: Measurement of the *rpiR* promoter activity in *B. cereus* WT or $\Delta casK/R$ mutant strains. Growth curves of the WT (black with empty symbols) and the $\Delta casK/R$ (grey with empty symbols) strains are represented at 12°C (A) and 37°C (B). β -galactosidase activity was measured in either the WT strain (black with full symbols) or in the $\Delta casK/R$ mutant (grey with empty symbols) harbouring pHT304-*rpiR/R'Z*, during growth at 12°C (A) and 37°C (B) in LB medium. Time 0 indicates the onset of stationary phase.

desA gene belong to CasK/R regulon

A lower proportion of FAs unsaturated by DesA was previously described in the FA profile of the $\Delta casK/R$ strain compared to that of the WT strain during growth at low temperature,

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suggesting a CasK/R-dependent role of DesA in the *B. cereus* cold adaptation (Diomandé *et al.*, submitted). In order to determine if *desA* belongs to the *casK/R* regulon we investigated on the expression of the *desA* promoter in both *B. cereus* WT and $\Delta casK/R$, using a transcriptional fusion between the *desA* promoter region and the *lacZ* reporter gene. *B. cereus* strains carrying the plasmids pHT304-*desA*'Z were cultured in LB medium at 12°C, and β -galactosidase activity was quantified all along the growth of the strains (Figure 6A). The β -galactosidase activity directed by the *desA* promoter was detected as early as the beginning of the exponential phase of growth for the WT strain, and then increased regularly over time.

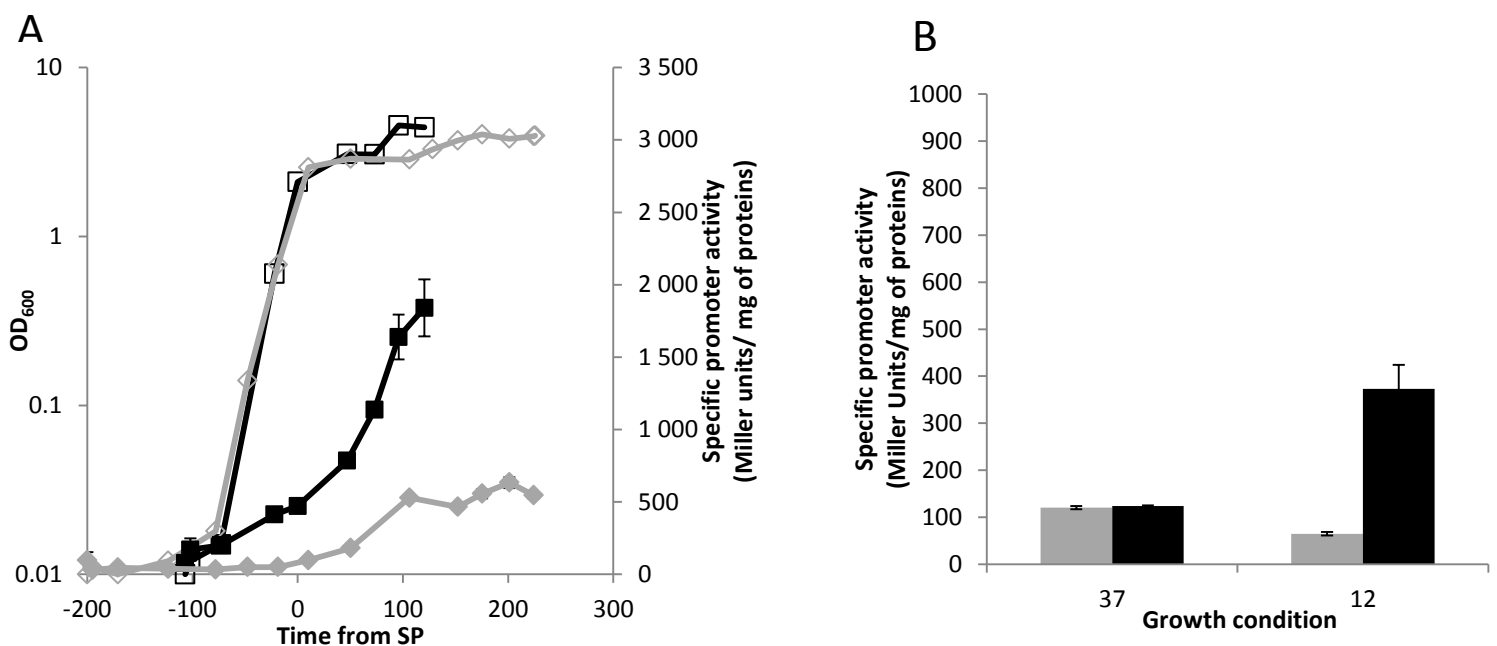


Figure 6: The *desA* promoter expression in *B. cereus* WT or $\Delta casK/R$ mutant strains. Growth curves of the WT (black with empty symbols) and the $\Delta casK/R$ (grey with empty symbols) strains are represented at 12°C (A) in LB medium. β -galactosidase activity was measured in either the WT strain (black with full symbols) or in the $\Delta casK/R$ mutant (grey with empty symbols) harbouring pHT304-*desA/R*'Z, during growth at 12°C (A) in LB medium. Time 0 indicates the onset of stationary phase. Figure B shows the *desA* promoter activity in exponential phase in the WT (black) and the $\Delta casK/R$ (grey) strains at 37°C and 12°C. Bars represent standard deviation. Mean and standard deviation values were calculated for triplicate measurements.

In the $\Delta casK/R$ strain, a delay of about 80h after the beginning of the exponential phase of growth occurred before the detection of a *PdesA* directed β -galactosidase activity (Figure 6A). This activity then increased regularly over time, but to a lesser extent than that observed for the WT strain (Figure 6A). Thus, the *desA* promoter activity was always significantly higher in the WT strain compared to the $\Delta casK/R$ strain ($p < 0.05$, Student's t-test). For instance, a

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specific activity of 35 ± 4.41 Miller Units/mg of protein was observed in the $\Delta casK/R$ strain vs 243 ± 6.67 Miller Units/mg of protein for the WT strain at EP entry. Such a difference was still observed at entry to SP between the two strains, 102.50 ± 12.66 Miller Units/mg of protein for the $\Delta casK/R$ strain but 408.33 ± 7.63 Miller Units/mg of protein for the WT strain. All these data indicate that the *desA* promoter regulation at low temperature partly depends on CasK/R. In order to see if this regulation was temperature-dependent, we quantified the *desA* promoter activity at optimal growth temperature, 37°C and at 12°C for the two strains during exponential growth phase (Figure 6B). No significant difference of activity was observed at 37°C between the WT and the $\Delta casK/R$ strain, in contrast to what was observed at 12°C.

Exogenous source of UFA modulate the expression of the *desA* gene

Previously we showed that supplementation of the growth medium with a UFAs source led to a lower proportion of $\Delta 5$ -UFAs (presumably synthesized by DesA) in *B. cereus*. We thus investigated on the effect of exogenous sources of UFAs on the *desA* promoter activity at low temperature. The lag phase representing the growth phase for adaptation to stress adaptation, we therefore quantified the *desA* promoter activity during this phase at low temperature in the WT and the $\Delta casK/R$ strains (Figure 7). During lag phase, 24h after the incubation at low temperature, a difference between the *desA* promoter activity in the WT and the mutant strains was already observed in the medium without any supplementation. When the medium was supplemented with exogenous UFAs source, the difference in promoter activity between the strains was almost abolished. In addition, a decrease of the *desA* promoter activity was observed after supplementation (Figure 7), suggesting that the synthesis of UFAs via DesA is no longer required

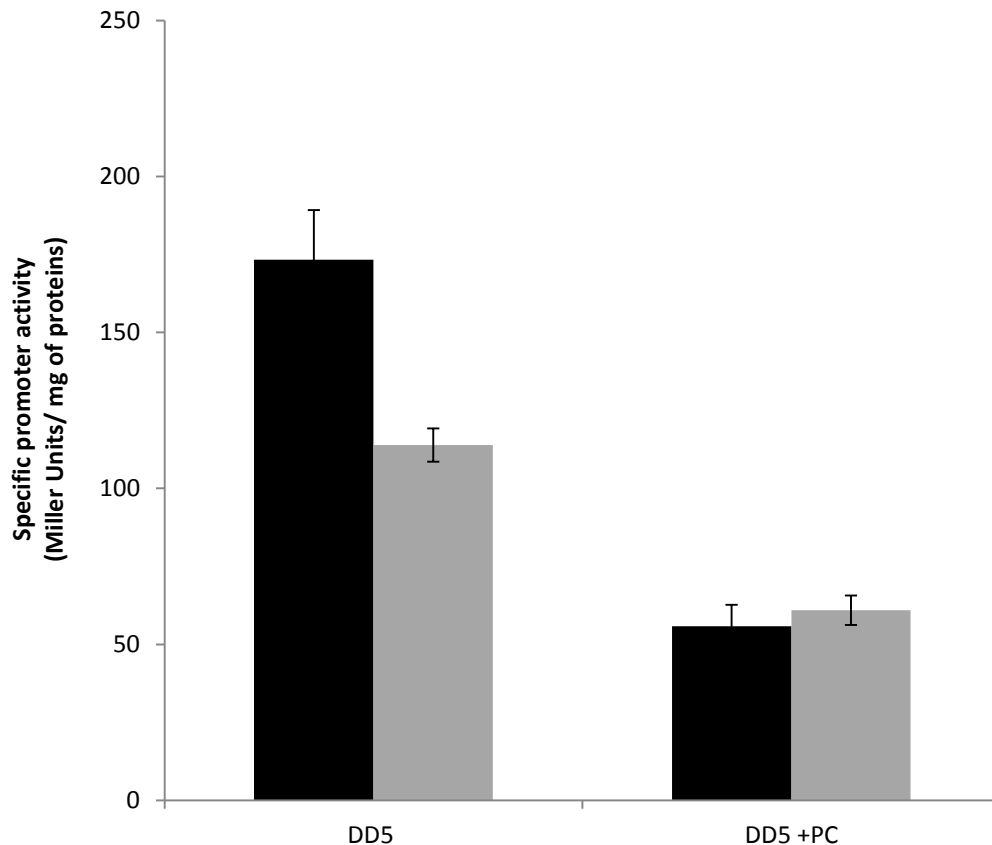


Figure 7: The *desA* promoter expression in *B. cereus* WT or $\Delta casK/R$ mutant strains with or without supplementation with a UFAs source. The beta-galactosidase activity was measured, in the WT (black) and the $\Delta casK/R$ (grey) strains harbouring pHT304-*desK/R*'Z during the lag phase at 12°C. The *desA* promoter activity was measured in LB medium (LB) and LB medium supplemented with a UFAs source (LB+UFAs source). Bars represent standard deviation. Mean and standard deviation values were calculated for triplicate measurements

CasR indirectly regulates the *casK/R*, *rpiR* and *desA* promoters

We performed Electrophoretic mobility shift assays to determine whether the CasK/R-dependent regulation of *casK/R*, *rpiR* and *desA* promoters described above was direct or indirect. Before this experiment CasR protein was purified and we investigated on role of the *in vitro* phosphorylation of CasR (see materials and methods section). The non-phosphorylated but His-tag-cleaved CasR protein and the phosphorylated but non-His-tag-cleaved CasR were loaded as controls in the same gel as the phosphorylated and his-tag-cleaved CasR sample (Figure 8-A). The non-phosphorylated but His-tag-cleaved CasR protein displayed one band at the size corresponding to a monomer of CasR protein e.g. 24 kDa on phostagTM gel (figure 8A). When the protein was phosphorylated and His-tag-cleaved, a band at a size of 48 kDa was observed suggesting that a dimer of CasR protein was formed. The

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phosphorylated CasR but non-His-tag-cleaved control profile displayed the two bands with a higher proportion for the monomer band. This indicated that both the phosphorylation and the cleavage of the His-tag of the purified CasR protein were necessary for an optimal dimerization of CasR.

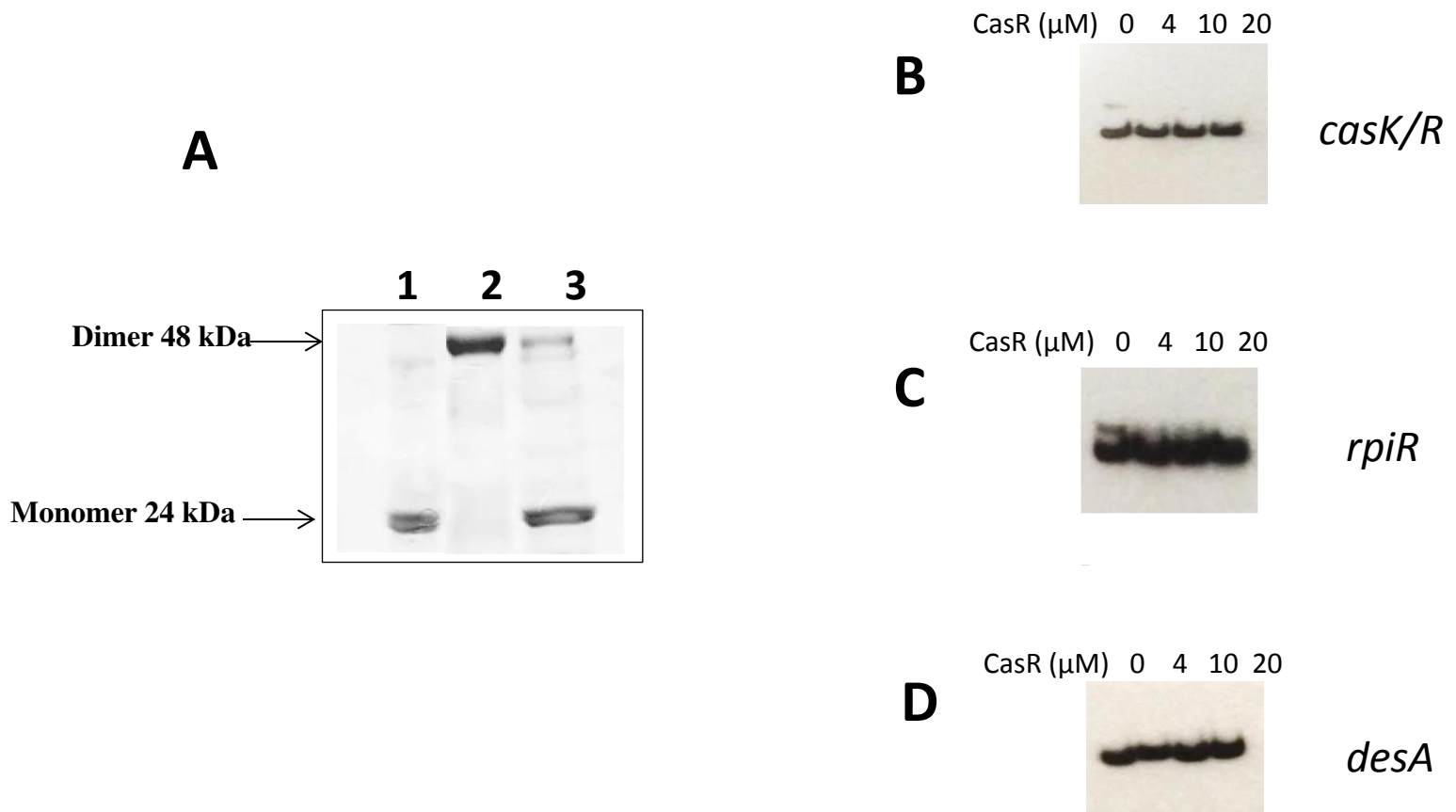


Figure 8: Verification of the CasK/R phosphorylation in vitro (A). 1 : non phosphorylated but His-tag-cleaved CasR ; 2 : phosphorylated and His-tag-cleaved CasR; 3 : phosphorylated but non-his-tag-cleaved CasR.

EMSA of different concentration of CasR in presence of the *casK/R* (B), *rpiR* (C) and *desA* (D) promoters is represented.

In order to determine if the CasK/R-dependent regulation of *casK/R*, *rpiR* and *desA* promoters described above was direct or indirect, we thus performed electrophoretic mobility shift assays (EMSA) with the phosphorylated and his-tag-cleaved CasR in presence of the *casK/R*, *rpiR* and *desA* promoters (Figure 8-B, 8-C, 8-D respectively). Different concentrations of CasR were tested, up to 20 μM. But, whatever the tested promoter region and the

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concentration of CasR, no shift was observed, suggesting that no direct binding between CasR and these promoters occurred.

Discussion

CasK/R was previously described for its involvement in the *B. cereus sensu lato* cold adaptation and in the UFAs composition change observed during low temperature adaptation (Diomandé et al., 2014) (Diomande *et al.*, submitted). The current study provided new insights about the regulation and the expression of this TCS.

As described for the majority of the TCS encoding genes (Szurmant and Hoch, 2010), *casK* and *casR* are chromosomally adjacent in *B. cereus* ATCC 14579. RT-PCR experiments showed that *casK* and *casR* were co-transcribed. Moreover, RT-PCR experiments also revealed that the *rpiR* gene, a gene encoding a putative RpiR-like regulator, located downstream the *casK/R* genes, belongs to the *casK/R* operon.

TCS are usually organized in operonic structures with genes involved in the same physiological function (Buelow and Raivio, 2010). Few studies focused on the role of RpiR in bacteria. The first RpiR protein was identified in *Escherichia coli* (Sorensen and Hove-Jensen, 1996) and other members of the RpiR family have been identified in both Gram-negative and Gram-positive bacteria and they usually act as transcriptional repressors or activators of sugar catabolism (Daddaoua et al., 2009, Yamamoto et al., 2001, Zhu et al., 2011, Jaeger and Mayer, 2008). Other roles are also dedicated to RpiR-like proteins, such as the regulation of purine catabolism (Guzman et al., 2013), the regulation of cell-to cell signaling for virulence (Tipton et al., 2013), the regulation of effector such as the RNAIII (Zhu et al., 2011), the regulation for high pressure resistance (Considine et al., 2011). The belonging of *rpiR* to the *casKR* region, conserved among *B. cereus* group (Diomandé et al., 2014), suggests a yet unidentified role of RpiR most probably linked to the CasKR TCS.

We wondered how the *casK/R* operon was regulated at low temperature. An increase of the *casK/R* promoter activity was observed at the end of exponential phase at optimal or low temperature in the WT strain. The 10 fold higher activity observed in the $\Delta casK/R$ when compared to the WT strain suggested that in *B. cereus*, a negative regulation of the *casK/R* operon generally occurs at low temperature. This observation is unusual because as TCSs genes are usually positively regulated in a given environmental stress adaptation (Tao et al., Aguilar et al., 2001b, Jordan et al., 2006). It remained to be determined if this negative

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regulation of CasK/R on its own promoter is direct, as suggested for about one third of TCS which directly regulates their own promoters (de Been et al., 2008), or indirect. To our knowledge, a negative regulation of a TCS expression mediated directly by the TCS itself has not been described. Only post translational or functional negative modulations of a TCS signaling pathway are described for *Pseudomonas syringae* RhpRS (Xiao et al., 2007), and the *Myxococcus xanthus* CrdSA (Willett and Kirby, 2011).

As an overexpression of *casK/R* in the $\Delta casK/R$ strain was observed, we wondered if it may impact the cold growth ability of *B. cereus*. Surprisingly, the cold growth ability of the WT strain harboring an additional copy of the *casK/R* region on a plasmid was reduced compared to that of the parental strain. A similar case was described for the *Pseudomonas syringae* RhpRS TCS which promotes induction of type III secretion system necessary during the interaction with plant, and for which overexpression results in the abolishment of this induction (Xiao et al., 2007). Our results suggest that the *casK/R* expression has to be finely regulated for optimal cold growth ability.

Our study also revealed the presence of a second promoter for the gene encoding the RpiR-like protein, located in the *casR rpiR* intergenic region. This promoter activity increased over time during at low- or optimal temperature. The 2 fold higher promoter activity observed in the $\Delta casK/R$ strain suggested a negative regulation of the *rpiR* gene whatever the growth temperature, and suggested that the *rpiR* gene belong to the *casK/R* regulon.

Consequently, the two promoters allowing the expression of *rpiR* are repressed in presence of *casK/R*. This may be explained by the fact that RpiR regulators often act as repressors (Daddaoua et al., 2009, Sorensen and Hove-Jensen, 1996, Tipton et al., 2013) and that their expression would be repressed when the cell does not need.

The *rpiR* gene was repressed in a CasK/R-dependent but in a temperature-independent manner suggesting that CasK/R also plays a role in *B. cereus* during optimal growth temperature by controlling the *rpiR* expression. The role of this member of the CasK/R regulon needs to be investigated by further studies.

Transcriptomic data previously identified the *desA* gene as a member of the CasK/R regulon (Diomandé et al. submitted). In the current study, expression of this gene was confirmed to be activated during growth at low temperature in a CasK/R-partially dependent manner. As DesA is involved in the metabolism of UFAs and that RpiR-like regulators have exclusively been described as metabolism regulators, we cannot exclude that RpiR may be involved in

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regulation of the FA metabolism where *rpiR* expression would be repressed by CasK/R to favor low temperature adaptation.

EMSA did not revealed direct interaction between CasR and the *casK/R*, *rpiR* or *desA* promoters. CasR displays an HTH domain for DNA binding, and thus could act as a transcriptional regulator. We may raise an hypothesis where CasR would directly regulate expression of other regulator(s) which in turn regulate the expression of genes of the CasK/R regulon.

Exogenous UFAs source were previously shown to restore the $\Delta casK/R$ strain growth ability at low temperature (Diomandé et al, submitted). We showed that in presence of exogenous FAs source, the *desA* promoter activity was reduced at low temperature. This suggested that in the abundant presence of UFAs, some are integrated in the bacterial cell and then reduces the need for additional molecules of the DesA enzyme because new unsaturation of FAs is not required. This also confirms the key role of UFAs for *B. cereus* cold adaptation.

Our study here revealed the existence of the *casK/R* operon including an *rpiR*-like gene. This operon expression is thoroughly regulated by CasK/R in a negative manner; something rarely observed when a TCS regulates its own expression. More investigations should be done in order to reveal the direct partners of CasK/R and may help in deciphering the role of this original TCS in *B.cereus* adaptation.

Acknowledgements

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Chapitre 5 :

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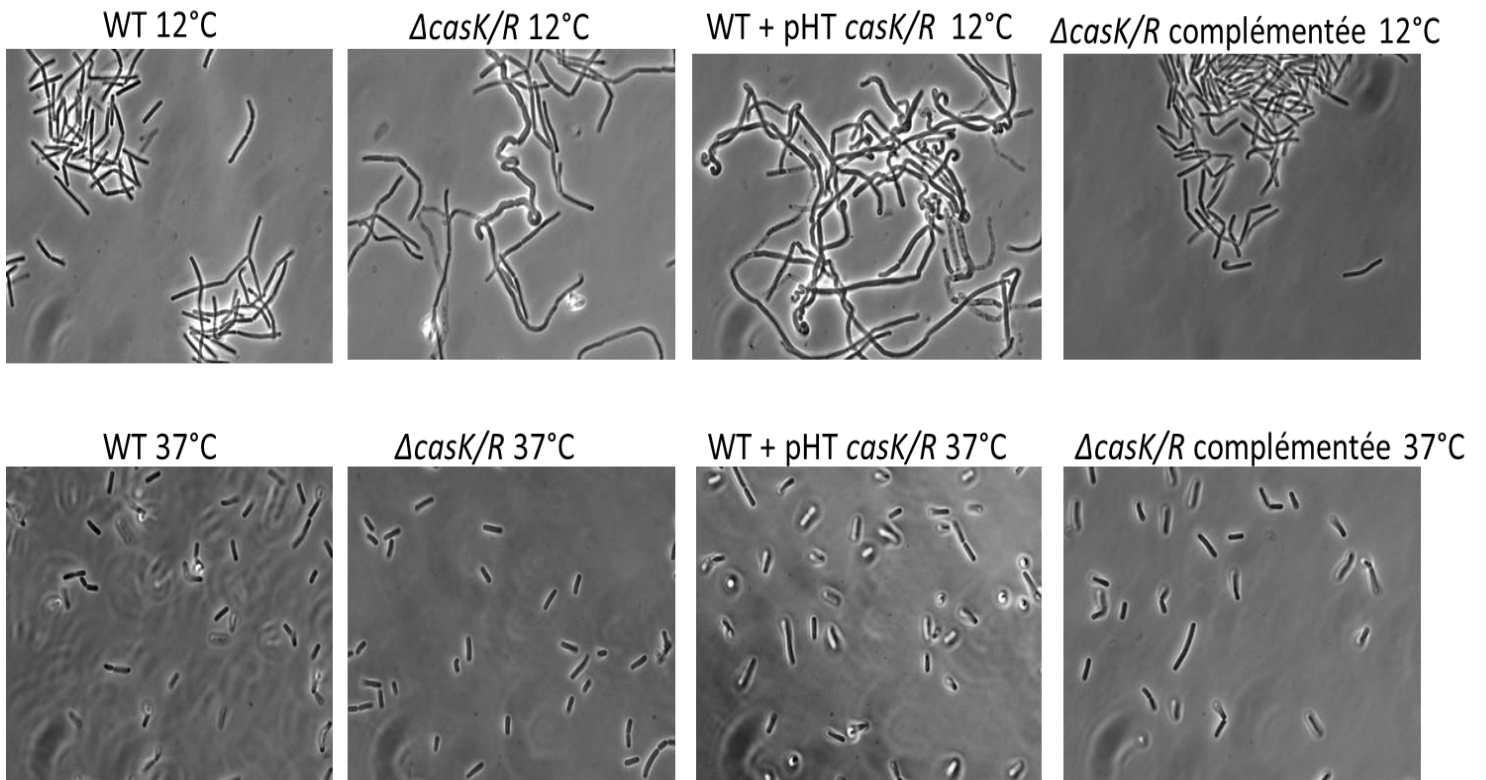
1- La morphologie de *B. cereus sensu lato* au froid

Comme précédemment énoncé, un des phénotypes caractérisant la croissance de *B. cereus* au froid est le changement morphologique de ces bactéries dans cette condition (Diomandé et al., 2014, de Sarrau et al., 2012c). La mutation d'un gène impliqué dans l'adaptation au froid ou des conditions drastiques de croissance tels que le froid combiné à l'anaérobiose peuvent accentuer ce phénotype (Pandiani et al., 2010a, Diomandé et al., 2014, de Sarrau et al., 2012c). Dans cette partie du chapitre 5, les modifications morphologiques liées à la température et à la mutation du gène *casK/R* vous sont présentées.

Les différentes souches de *Bacillus cereus sl* utilisées lors de cette étude ont été observées au microscope optique à contraste de phase. Des observations réalisées en phase stationnaire de croissance, ont montré un allongement des cellules des souches sauvages et mutantes lorsqu'elles sont cultivées à basse température.

Quelle que soit la souche de *Bacillus cereus* considérée, les cellules à 37 °C ont le même aspect (Figure S1). Cependant, une différence entre la souche WT et $\Delta casK/R$ est visible au froid. Ce phénotype est bien décrit dans le chapitre 1, pour la souche ATCC14579. Les cellules de la souche $\Delta casK/R$ sont plus ondulées et quelque fois plus allongées que les cellules WT à 12°C.

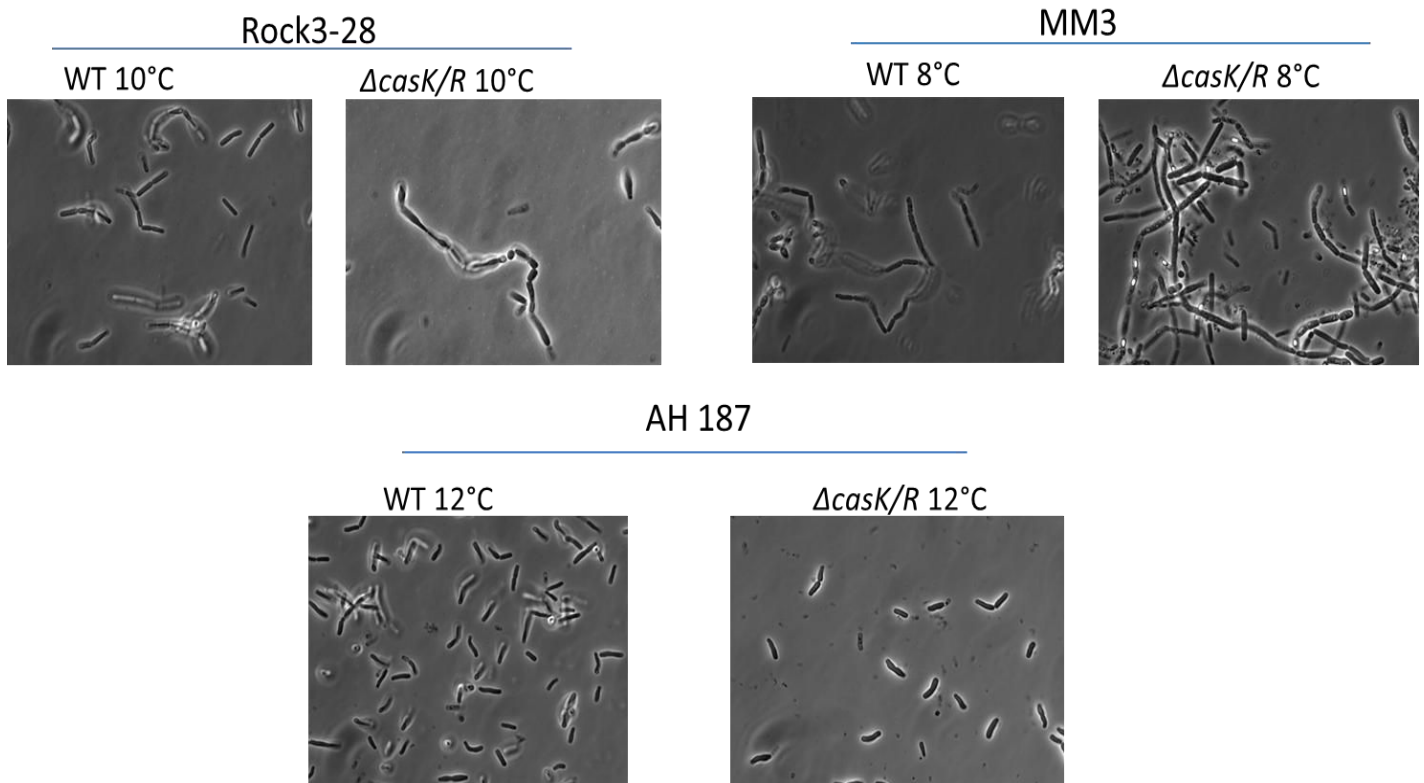
Lorsque le mutant $\Delta casK/R$ est complémenté, la souche complémentée présente une croissance et une morphologie identiques à celles de la souche WT. Nous avons cependant observé que si l'on introduit le plasmide portant les gènes *casKR* dans la souche WT (WT+pHT*casK/R*), celle-ci perd son capacité à se développer au froid et présente une morphologie modifiée, tout comme la souche mutante $\Delta casK/R$ (Figure S1).



Figures S1 : Séries de photos des différentes souches de *B. cereus* ATCC 14579 observées au microscope optique à contraste de phase (grossissement x 1000)

Une différence entre les souches WT et $\Delta casK/R$ au froid est aussi observée pour les autres souches du groupe *B. cereus sensu lato* testées. Les cellules $\Delta casK/R$ des souches Rock3-28 et MM3 sont plus boursoufflées et toujours plus allongées que les souches WT (Figure S2).

La souche AH187 fait cependant exception : en effet, à basse température, la souche AH 187 présente des bacilles semblables à ceux observés à température optimale que l'on considère la souche WT et $\Delta casK/R$ (résultats non présentés). Cette souche est la plus mésophile des souches de *B. cereus sl* testées et la T_{min} testée était donc relativement élevée (13°C vs 8°C pour les plus psychrotolérantes), ce qui pourrait expliquer cette différence phénotypique. L'allongement des cellules est observable autant chez les souches WT que $\Delta casK/R$ testées. Il pourrait s'agir d'un mécanisme alternatif facilitant l'adaptation au froid des souches décrites et absent chez la souche AH187.



Figures S2 : Séries de photos des différentes souches de *B. cereus sl* à basse température observées au microscope optique à contraste de phase (grossissement x 1000)

2- D'autres pistes sur le régulon de CasK/R

a) Un grand nombre de gènes est régulé par CasK/R

Dans l'optique de mieux comprendre le fonctionnement de CasK/R nous avons réalisé une analyse du transcriptome des souches WT et du $\Delta casK/R$, cela nous a permis d'identifier plusieurs pistes quant au régulon de CasK/R à basse température versus température optimale. En effet, cette étude transcriptomique a permis de mettre en évidence un grand nombre de gènes différentiellement exprimés dans ces conditions. Comme indiqué dans le chapitre 3, cette analyse des transcrits a été réalisée en début de phase exponentielle et en début de phase stationnaire. Près de 500 gènes étaient différentiellement exprimés en début de phase exponentielle et plus de 1000 gènes en phase stationnaire (Figure S3). En combinant les 2 conditions il demeure 148 gènes différentiellement exprimés en début de phase exponentielle et phase stationnaire chez la souche $\Delta casK/R$ par rapport à la souche WT soit un grand nombre de gènes régulés par CasK/R. Cette observation nous a conduit à émettre l'hypothèse

que CasK/R pourrait être un système ayant un rôle pléiotrope et régulant donc un grand nombre de mécanismes différents, dont certains semblent jouer un rôle majeur dans l'adaptation au froid (i.e., régulation du métabolisme des acides gras, cf chapitre 3).

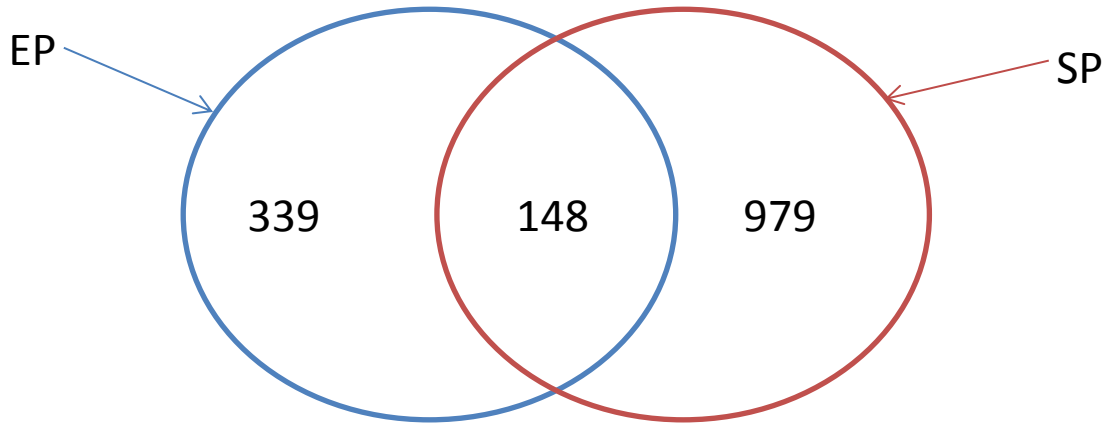


Figure S3 : Diagramme de Venn représentant les gènes exprimés en phase exponentielle (EP) et/ou en phase stationnaire (SP) de croissance

b) Le COG majoritaire régulé par CasK/R en début de phase exponentielle et phase stationnaire est celui du métabolisme

Parmi les 148 gènes différentiellement exprimés au froid dans les deux phases de croissance analysées, 62 gènes appartiennent à un COG majoritaire correspondant aux gènes du métabolisme (données supplémentaires chapitre 3). Parmi ces 62 gènes (Tableau S1), 50 sont sous-exprimés et 12 surexprimés chez la souche $\Delta casK/R$ comparée à la souche WT.

Comme indiqué dans le tableau S1, les systèmes de transports liés aux métabolismes sont majoritairement touchés par la mutation de *casK/R* (14 gènes). Le métabolisme des acides gras est également très affecté dans la souche mutante (12 gènes). Cette observation a fait l'objet de recherches développées dans le chapitre 3. On retrouve également, le métabolisme des acides aminés ainsi que la phosphorylation oxydative (voie de production d'ATP et régénération de cofacteurs) et le métabolisme des purines et celui de sucres (glucose, galactose) affectés par la mutation de *casK/R*. Toutes ces données laissent supposer que le métabolisme énergétique de la bactérie est affecté chez la souche $\Delta casK/R$.

Le métabolisme énergétique est réglé à basse température pour que le rendement énergétique des cellules soit au plus bas durant cette condition de stress (De Maayer et al., 2014). De plus, le niveau d'ATP est aussi connu pour augmenter lors de l'adaptation à basse température, par rapport aux conditions optimales, pour permettre aux réactions

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enzymatiques, ralenties au froid, d'avoir lieu (Napolitano and Shain, 2004). L'observation faite ici pour la souche $\Delta casK/R$ suggèrerait donc un métabolisme plus ralenti chez cette souche, ce qui se traduirait par le retard de croissance observée à basse température comparée à la souche WT. Un réseau de régulation impliquant CasK/R pourrait expliquer cette différence et expliquerait le si grand nombre de gènes différentiellement exprimés entre les 2 souches identifiés par l'analyse du transcriptome.

Tableau S1 : Liste des 62 gènes différentiellement exprimés chez la souche WT par rapport à la souche $\Delta casK/R$ en début de phase exponentielle (EP) et phase stationnaire (SP) à basse température

| Numéro de locus | fonction putative | Catégorie de COG | Métabolisme concerné | alias | EP | SP |
|-----------------|---|------------------|--------------------------------------|-------|---------|---------|
| | | | | | fold | fold |
| BC2311 | 4'-phosphopantetheinyl transferase | métabolisme | 4'-phosphopantetheinyl transferase ? | | 0.4591 | 0.52745 |
| BC2980 | Arginine permease | métabolisme | ACIDES AMINES | rocC | 0.20825 | 0.3953 |
| BC2209 | D-alanine aminotransferase | métabolisme | ACIDES AMINES | | 0.27885 | 0.28085 |
| BC3550 | Argininosuccinate lyase | métabolisme | ACIDES AMINES | | 0.29375 | 0.334 |
| BC4369 | Dimethyladenosine transferase | métabolisme | ACIDES AMINES | yrT | 0.31185 | 0.51615 |
| BC4427 | Prephenate hydratase | métabolisme | ACIDES AMINES | pheA | 0.4385 | 0.5277 |
| BC5406 | O-acetylhomoserine sulfhydrilase | métabolisme | ACIDES AMINES | | 0.2116 | 0.3149 |
| BC2289 | 3-hydroxyisobutyrate dehydrogenase | métabolisme | ACIDES AMINES | | 0.4277 | 0.295 |
| BC4906 | Aspartate aminotransferase | métabolisme | ACIDES AMINES | patB | 3.93325 | 3.6643 |
| BC0400 | Fatty acid desaturase | métabolisme | ACIDES GRAS | | 0.1499 | 0.53625 |
| BC1088 | Long-chain-fatty-acid--CoA ligase | métabolisme | ACIDES GRAS | yhfL | 0.3847 | 0.35795 |
| BC1173 | 3-oxoacyl-[acyl-carrier-protein] synthase III | métabolisme | ACIDES GRAS | | 0.2806 | 0.38085 |
| BC1174 | 3-oxoacyl-[acyl-carrier-protein] synthase | métabolisme | ACIDES GRAS | fabF | 0.27415 | 0.43265 |
| BC1216 | enoyl-[acyl-carrier-protein] reductase (fabL) (NADPH) | métabolisme | ACIDES GRAS | fabI | 0.3214 | 0.5369 |
| BC1312 | 3-hydroxybutyryl-CoA hydratase | métabolisme | ACIDES GRAS | | 0.47875 | 0.4464 |
| BC2288 | Acyl-CoA dehydrogenase | métabolisme | ACIDES GRAS | | 0.44505 | 0.32455 |
| BC2983 | delta5 acyl-lipid desaturase | métabolisme | ACIDES GRAS | | 0.2104 | 0.19655 |
| BC3630 | Medium-chain-fatty-acid--CoA ligase | métabolisme | ACIDES GRAS | | 0.24145 | 0.4693 |
| BC4524 | 3-hydroxybutyryl-CoA hydratase | métabolisme | ACIDES GRAS | ysiB | 0.26765 | 0.3255 |

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| | | | | | | |
|--------|---|-------------|---|------|---------|----------|
| BC5344 | Acetyl-CoA acetyltransferase | métabolisme | ACIDES GRAS | mmgA | 0.4154 | 0.5528 |
| BC5426 | Acyl-CoA hydrolase | métabolisme | ACIDES GRAS | ykhA | 2.8356 | 3.60145 |
| BC2285 | 2-methylcitrate synthase | métabolisme | CYCLE DE KREBS | mmgD | 0.2924 | 0.2941 |
| BC0413 | Exo-alpha-1,4-glucosidase | métabolisme | GALACTOSE | yugT | 0.50585 | 0.4661 |
| BC2307 | Glycine-AMP ligase | métabolisme | Glycine-AMP ligase? | | 0.21475 | 0.32435 |
| BC3555 | Aldehyde dehydrogenase | Métabolisme | GLYCOLYSE | dhaS | 0.32525 | 0.4458 |
| BC1426 | Uroporphyrin-III C-methyltransferase | métabolisme | METAB DES PORPHYRINES | ylnD | 2.6262 | 35.7123 |
| BC2432 | Methyltransferase | métabolisme | Methyltransferase ? | ybaJ | 3.47905 | 9.377 |
| BC4340 | NAD(P)H nitroreductase | Métabolisme | NAD(P)H nitroreductase ? | ydfN | 2.8822 | 15.83465 |
| BC4014 | Neopullulanase | métabolisme | Neopullulanase? | yvfK | 0.4609 | 0.20515 |
| BC1036 | Glycerol-3-phosphate dehydrogenase | Métabolisme | PHOSPHOLIPIDES | glpD | 0.29535 | 0.36555 |
| BC1523 | Menaquinol-cytochrome c reductase cytochrome b subunit | Métabolisme | PHOSPHORYLATION OXYDATIVE | | 0.4867 | 0.5434 |
| BC3943 | Cytochrome c oxidase polypeptide I | Métabolisme | PHOSPHORYLATION OXYDATIVE | ctaD | 0.4455 | 0.27165 |
| BC3944 | Cytochrome c oxidase polypeptide II | Métabolisme | PHOSPHORYLATION OXYDATIVE | | 0.4248 | 0.28475 |
| BC5300 | NADH-quinone oxidoreductase chain B | Métabolisme | PHOSPHORYLATION OXYDATIVE | | 0.26485 | 0.44105 |
| BC5301 | NADH-quinone oxidoreductase chain A | Métabolisme | PHOSPHORYLATION OXYDATIVE | | 0.28325 | 0.4347 |
| BC4523 | Electron transfer flavoprotein beta- subunit | Métabolisme | PHOSPHORYLATION OXYDATIVE RESPIRATION | etfA | 0.3373 | 0.39705 |
| BC2306 | Glycine-AMP ligase | métabolisme | (PSEUDOGENE) | dhbF | 0.2634 | 0.4428 |
| BC0329 | Phosphoribosylformylglycinamide synthase | métabolisme | PURINES | purL | 0.44175 | 0.54045 |
| BC1422 | Sulfate adenylyltransferase | métabolisme | PURINES | | 2.5704 | 48.3881 |
| BC1423 | Adenylylsulfate kinase | métabolisme | PURINES | yisZ | 2.5282 | 39.4034 |
| BC0215 | Oligopeptide-binding protein oppA | métabolisme | RESISTANCE ANTIBIOTIQUES | | 0.10065 | 0.46595 |
| BC0204 | Bicyclomycin resistance protein | métabolisme | RESISTANCE ANTIBIOTIQUES | ydgK | 0.42335 | 0.4504 |
| BC3586 | Oligopeptide-binding protein oppA | métabolisme | RESISTANCE ANTIBIOTIQUES | | 0.21235 | 0.4744 |
| BC2304 | 2,3-dihydroxybenzoate-AMP ligase | métabolisme | SIDEROPHORES | dhbE | 0.22005 | 0.384 |
| BC2305 | Isochorismatase | métabolisme | SIDEROPHORES | dhbB | 0.2693 | 0.49985 |

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| | | | | | | |
|--------|---|-------------|---------------------------------------|------|---------|---------|
| BC1421 | Phosphoadenosine phosphosulfate reductase | métabolisme | SOUFFRE | cysH | 2.8406 | 18.1825 |
| BC2303 | Isochorismate synthase | métabolisme | SYNTH DES UBIQUINONES | dhbC | 0.21025 | 0.46105 |
| BC2300 | Oxalate/formate antiporter | métabolisme | TRANSPORT | | 0.39435 | 0.26395 |
| BC3727 | Formate transporter | métabolisme | TRANSPORT | yrhG | 2.87045 | 4.7447 |
| BC3644 | Sodium/proline symporter | métabolisme | TRANSPORT | opuE | 2.99695 | 3.66725 |
| BC4011 | Cyclodextrin transport system permease protein | métabolisme | TRANSPORT ABC TRANSPORT | yvfM | 0.39265 | 0.2153 |
| BC0346 | ABC transporter ATP-binding protein | métabolisme | TRANSPORT ABC TRANSPORT METHIONINE | | 0.24125 | 0.4267 |
| BC0347 | ABC transporter permease protein | métabolisme | TRANSPORT ABC TRANSPORT METHIONINE | | 0.1852 | 0.4361 |
| BC0348 | ABC transporter substrate-binding protein | métabolisme | TRANSPORT ABC TRANSPORT METHIONINE | yhcJ | 0.25265 | 0.49855 |
| BC1838 | Copper resistance protein | métabolisme | TRANSPORT CUIVRE | ycnJ | 0.44815 | 0.33715 |
| BC0807 | PTS system, diacetylchitobiose-specific IIA component | métabolisme | TRANSPORT DE SUCRE | | 0.16895 | 0.45455 |
| BC0809 | PTS system, diacetylchitobiose-specific IIC component | métabolisme | TRANSPORT DE SUCRE | | 0.26665 | 0.23075 |
| BC0516 | LCTB protein | métabolisme | TRANSPORT POTASSIUM | | 2.62485 | 3.98375 |
| BC0482 | PTS system, N-acetylglucosamine-specific IIBC component | métabolisme | TRANSPORT POUR SYNTHESE ACIDES AMINES | nagP | 0.3515 | 0.1277 |
| BC5211 | PTS system, lichenan oligosaccharide-specific IIC component | métabolisme | TRANSPORT SUCRE | | 0.2029 | 0.39315 |
| BC0847 | Transporter, Drug/Metabolite Exporter family | métabolisme | TRANSPORT? | | 0.21195 | 0.3694 |
| BC4502 | Xanthine permease | métabolisme | Xanthine permease? | | 3.2951 | 2.6654 |

c) Qu'en est-il des régulateurs ?

Un certain nombre de régulateurs sont différentiellement exprimés chez la souche $\Delta casK/R$ par rapport à la souche WT au froid et sont listés dans le tableau S2. Ils appartiennent au COG « information storage and processing » ou stockage et traitement des informations.

Tableau S2 : liste des gènes appartenant au COG : « Information storage and processing » incluant les régulateurs régulés par CasK/R (en gras) en début de phase exponentielle (EP) et phase stationnaire (SP), $\Delta casK/R$ vs WT, à basse température

| | | | | EP | SP |
|--------|---|-------------|------------------------------------|--------|--------|
| locus | | | | | |
| number | putative function | alias | Broad COG category (ies) | n-fold | n-fold |
| BC0004 | DNA replication and repair protein <i>recF</i> | <i>recF</i> | Information storage and processing | 0.42 | 0.19 |
| BC0005 | DNA gyrase subunit B | | Information storage and processing | 0.42 | 0.21 |
| BC0006 | DNA gyrase subunit A | <i>gyrA</i> | Information storage and processing | 0.32 | 0.43 |
| BC0473 | Arginine utilization regulatory protein <i>rocR</i> | <i>rocR</i> | Information storage and processing | 0.48 | 0.47 |
| BC0806 | Transcription antiterminator, BglG family | <i>licR</i> | Information storage and processing | 0.31 | 0.38 |
| BC2979 | Transcriptional regulator, IclR family | | Information storage and processing | 0.23 | 0.35 |
| BC3792 | Transcriptional regulator, GntR family | <i>ymfC</i> | Information storage and processing | 0.20 | 0.52 |
| BC4525 | Transcriptional regulator, TetR family | <i>ysiA</i> | Information storage and processing | 0.32 | 0.41 |
| BC2218 | Transcriptional regulator, RpiR family | <i>ybbH</i> | Information storage and processing | 2.97 | 6.98 |
| BC4320 | SSU ribosomal protein S20P | <i>rpsT</i> | information storage and processing | 3.78 | 3.66 |
| BC4902 | Transcriptional regulator, AsnC family | <i>alaR</i> | Information storage and processing | 5.70 | 3.46 |

Huit régulateurs sont listés dans ce tableau. Le gène BC2979 code pour un régulateur de type IclR, ces régulateurs qui peuvent être des répresseurs ou des activateurs, régulent le catabolisme de molécules telles que le L-gluconate, le gentisate, l'hydroxybenzoate (Shimizu and Nakamura, 2014, Chao and Zhou, 2014). Le gène BC3792 code pour un régulateur de type GntR, ces régulateurs peuvent être des répresseurs ou des activateurs régulant par exemple le transport de phosphate ainsi que l'utilisation des acides aminés (Gebhard et al., 2014, Gaugue et al., 2014). Le gène *fadR* (BC4525) décrit dans les chapitres 1 et 3 de ce manuscrit est un répresseur du catabolisme des acides gras (Matsuoka et al., 2007). BC0806

est un gène codant putativement pour un activateur de l'opéron *lic* jouant un rôle dans le transport et la dégradation des sucres (Tobisch et al., 1999). BC0473 serait un gène codant pour un activateur de type RocR de la dégradation des acides aminés (Zaprasis et al., 2014). Les régulateurs de type AsnC sont impliqués dans plusieurs types de régulations, tels que le transport du chromate (Aguilar-Barajas et al., 2013), le métabolisme des acides gras ou plusieurs autres voies physiologiques (Deng et al., 2011, Dey and Ramachandran, 2014, Kölling et al., 1988). Le gène BC2218, codant pour le régulateur RpiR-like en aval de *casK/R* est décrit dans le chapitre 4 de ce manuscrit. Les régulateurs RpiR-like sont généralement des répresseurs du catabolisme des sucres.

Le gène *rpsT* (BC4320) coderait pour une sous-unité ribosomale très peu décrite.

D'une manière générale, ces régulateurs semblent cibler différents métabolismes. Ceci conforte l'hypothèse d'un rôle majeur de CasK/R dans différents métabolismes à basse température.

Tout ceci représente un ensemble de pistes à creuser pour découvrir d'éventuels partenaires aux protéines du TCS CasK/R qui seraient impliqués dans l'adaptation aux basses températures.

d) Des gènes liés à la sporulation sont régulés par CasK/R

Parmi les 148 gènes énoncés précédemment, 6 sont liés à la sporulation chez *B. cereus* (Tableau S3). Le gène codant pour la kinase de phosphorylation KinB, nécessaire à l'initiation de la sporulation est sous exprimé chez la souche $\Delta casK/R$ par rapport à la souche WT (Tojo et al., 2013). Les 5 autres gènes sont surexprimés chez la souche mutante. GerE est une protéine régulatrice dont le rôle est d'assurer la synthèse optimale des protéines de la tunique pour produire des spores compétentes à la germination (Ichikawa et al., 1999). Les autres gènes sont très peu étudiés dans la littérature. Cette observation est particulièrement intéressante d'autant plus que nous avons réalisé des essais de sporulations des souches WT et $\Delta casK/R$ chez la souche ATCC 14579 en milieu de sporulation (CCY), à 30°C et dans ces conditions la souche $\Delta casK/R$ présentait un retard de 24h au niveau de l'initiation de sporulation.

Il serait intéressant d'étudier l'implication de CasK/R dans les processus de sporulation et de germination de *B. cereus* à basse température de façon à comprendre pourquoi ces gènes sont régulés par CasK/R au froid.

Chapitre 5 : Suppléments d'informations

Tableau S3 : liste des gènes liés à la spore et régulés par CasK/R ($\Delta casK/R$ vs WT), en début de phase exponentielle (EP) et phase stationnaire (SP) à basse température

| | | | | EP | SP | |
|--------|----------------------------|-------|--------------------------|------|-------|--|
| locus | | | | | | |
| number | putative function | alias | Broad COG category (ies) | fold | fold | |
| | | | cellular process and | | | |
| BC4007 | Sporulation kinase B | kinB | signaling | 0.27 | 0.36 | |
| BC0390 | Spore coat protein B | | poorly characterized | 3.21 | 6.64 | |
| | Spore germination protein | | | | | |
| BC0704 | LA | | poorly characterized | 9.40 | 3.47 | |
| BC1470 | Spore maturation protein A | spmA | poorly characterized | 7.35 | 5.42 | |
| BC4495 | Germination protein germ | gerM | poorly characterized | 4.21 | 12.15 | |
| BC4501 | Germination protein gerE | gerE | poorly characterized | 3.24 | 2.36 | |

Conclusion générale et perspectives

Conclusions générale et perspectives

Beaucoup d'études témoignent des remarquables compétences qu'ont les bactéries pour s'adapter à divers changements environnementaux aussi brutaux soient-ils. Pour certains groupes de bactéries, comme *Bacillus cereus sensu lato*, ces capacités d'adaptations ont modélisé historiquement la diversification de niches écologiques présentement observés (Guinebretière et al., 2008).

L'espèce *B. cereus sensu stricto* comprend des pathogènes humains souvent isolés des aliments comme responsables de toxi-infection alimentaire collective (2^e cause en France en 2012, InVS), mais aussi responsables de l'altération des aliments. De plus, d'autres espèces plus psychrotolérantes de *Bacillus cereus sensu lato* sont capables de produire des toxines telles que la céréulide, responsable de syndrome de type émétique chez l'homme.

Les travaux de recherches détaillés dans ce manuscrit font partie intégrante d'un projet visant à mieux comprendre les mécanismes permettant une bonne adaptation des souches de *Bacillus cereus sensu lato* aux basses températures, dans le but d'une meilleure maîtrise des risques associés à ces souches pathogènes de *B. cereus*, tant mésophiles que psychrotolérants. L'adaptation au froid de *B. cereus* est connue pour être multifactorielle comme le témoignent les études réalisées sur ce pathogène (Mayr et al., 1996, Pandiani et al., 2010a, Pandiani et al., 2011a, Brillard et al., 2010c, de Sarrau et al., 2012c, de Sarrau et al., 2013d). Les TCS représentent un des principaux mécanismes d'adaptation aux environnements des bactéries. Pourtant, au début de ce projet de recherche, aucun TCS n'avait été identifié dans l'adaptation de *B. cereus* au froid même si des approches sans a priori telles que l'étude d'une banque de mutant obtenue par insertion de transposon (Broussolle et al., 2010a) et une stratégie de piégeage de promoteurs ont été utilisées pour mettre en évidence les gènes impliqués dans l'adaptation de cette bactérie au froid.

Peu avant le début de ma thèse, le TCS YhcY/Z a été mis en évidence, lors d'une étude du transcriptome de la souche type de *B. cereus* ATCC 14579 au froid par rapport à la température optimale, comme surexprimé à basse température. La caractérisation du mutant $\Delta yhcY/Z$ chez la souche ATCC 14579 a permis de mettre en évidence que celui-ci était affecté dans sa capacité à croître et survivre à basse température. La morphologie de la bactérie mutée était aussi modifiée par rapport à celle de la souche WT. De plus, la complémentation

en *trans* de la souche mutante avec une copie de *casK/R*, celle-ci permettait de restaurer les phénotypes de la souche WT à basse température. Ainsi au vu de l'implication de ce TCS dans l'adaptation de *B. cereus* au froid, celui-ci a été renommé CasK/R, Cas pour « cold adaptation system ». L'analyse phénotypique des mutants réalisés chez des souches représentant la diversité de *B. cereus sensu lato* a permis de conclure que CasK/R avait un rôle plus générique dans l'adaptation au froid de *B. cereus sl*

Pour une meilleure compréhension du fonctionnement de ce TCS, une étude transcriptomique réalisée sur la souche type et son mutant isogénique $\Delta casK/R$ a été réalisée et a révélé que CasK/R régulait, entre autre, le métabolisme des acides gras à basse température. Nous avons donc choisi de nous focaliser sur l'impact de CasK/R sur le métabolisme des acides gras. Les profils d'acides gras réalisés suite à cette étude transcriptomique ont permis de déterminer que les AGIs de la membrane sont principalement augmentés lors de la croissance aux basses températures et plus particulièrement les AG insaturés par l'action de la désaturase DesA. Chez la souche $\Delta casK/R$, une plus faible proportion d'AG insaturés par DesA est retrouvée par rapport à la souche WT. Lors de cette étude, le rôle crucial des AGI dans la croissance au froid a été mis en évidence car la croissance au froid de la souche $\Delta casK/R$ est restaurée au même niveau que celle de la souche WT en présence de lécithine, une source d'AGI végétal présente dans certains aliments. Ceci a permis de mettre en évidence le rôle de CasK/R dans la régulation de la composition des acides gras membranaires pour l'adaptation au froid.

Avec le recul, avoir fait le choix de se focaliser sur le métabolisme des AGs s'est avéré assez fructueux, il aurait toutefois été aussi intéressant d'élucider le rôle de CasK/R dans d'autres types de métabolismes tels que celui des acides aminés ou des purines car des gènes appartenant à ces métabolismes faisaient aussi partie du régulon de CasK/R.

Dans la suite des travaux de recherches, plus d'informations ont pu être acquises sur l'expression de *casK/R* et les régulations associées à ce TCS. Les expériences de RT-PCR ont permis de mettre en évidence l'existence d'un opéron constitué de *casK*, *casR* ainsi que du gène en aval, *rpiR*. L'existence d'un promoteur individuel au gène *rpiR* a aussi été démontrée. CasR réprimerait les 2 promoteurs, celui gouvernant la transcription de l'opéron *casKR-rpiR*, et celui spécifique à *rpiR*, durant la croissance à basse température. La régulation négative d'un TCS sur son propre promoteur semble originale mais pas décrit dans la littérature à notre connaissance.

La répression dépendante de CasK/R, s'exerçant sur le promoteur du gène *rpiR* était aussi observée à 37°C suggérant que CasK/R est aussi actif à température optimale. Sur la base de la connaissance du rôle des régulateurs RpiR-like chez les bactéries (Daddaoua et al., 2009, Yamamoto et al., 2001, Zhu et al., 2011, Jaeger and Mayer, 2008), il paraît assez probable qu'à 37°C, CasK/R soit impliqué dans la régulation d'un métabolisme qui reste à identifier.

Contrairement à la transcription de l'opéron *casKR-rpiR*, et à celle spécifique à *rpiR*, mentionnées ci-dessus, la transcription de *desA* est positivement régulée par CasK/R.

Cependant l'expression de *desA* est diminuée en présence de sources d'AGIs exogènes à basse température, ceci suggère que chez *B. cereus* l'expression de *desA* est modulée en fonction de la disponibilité en acides gras dans le milieu de croissance. Ainsi, lorsqu'il y a suffisamment d'AGI incorporables dans la membrane de *B. cereus* pour son adaptation au froid, il n'est pas nécessaire de mobiliser DesA pour les insaturations des AGs.

Nos résultats mettent l'accent sur la capacité qu'a *B. cereus* à utiliser les sources d'acides gras de son environnement pour favoriser son développement à basse température comme précédemment démontré (de Sarrau et al., 2013d).

Les résultats de retards sur gels indiquent que la régulation exercée par CasR sur les promoteurs de *casK/R*, de *desA* et du gène *rpiR* n'est pas directe. La recherche de séquence consensus au niveau des régions promotrices nommés ci-dessus n'a par ailleurs pas permis de révéler de boîtes conservées permettant une possible fixation de CasR. Ceci suggère un intermédiaire de régulation c'est-à-dire un régulateur qui serait lui-même régulé par CasR avant de réguler les gènes nommés précédemment. Ce régulateur pourrait être l'un des régulateurs dont les gènes ont été identifiés dans le régulon de CasK/R et listés dans la partie chapitre 5-2c, à commencer par le régulateur RpiR qui s'avère être un candidat qui mériterait d'être étudié.

Le fonctionnement de CasK/R à basse température peut être synthétisé à l'aide des deux schémas ci-dessous (Figures S4 et S5)

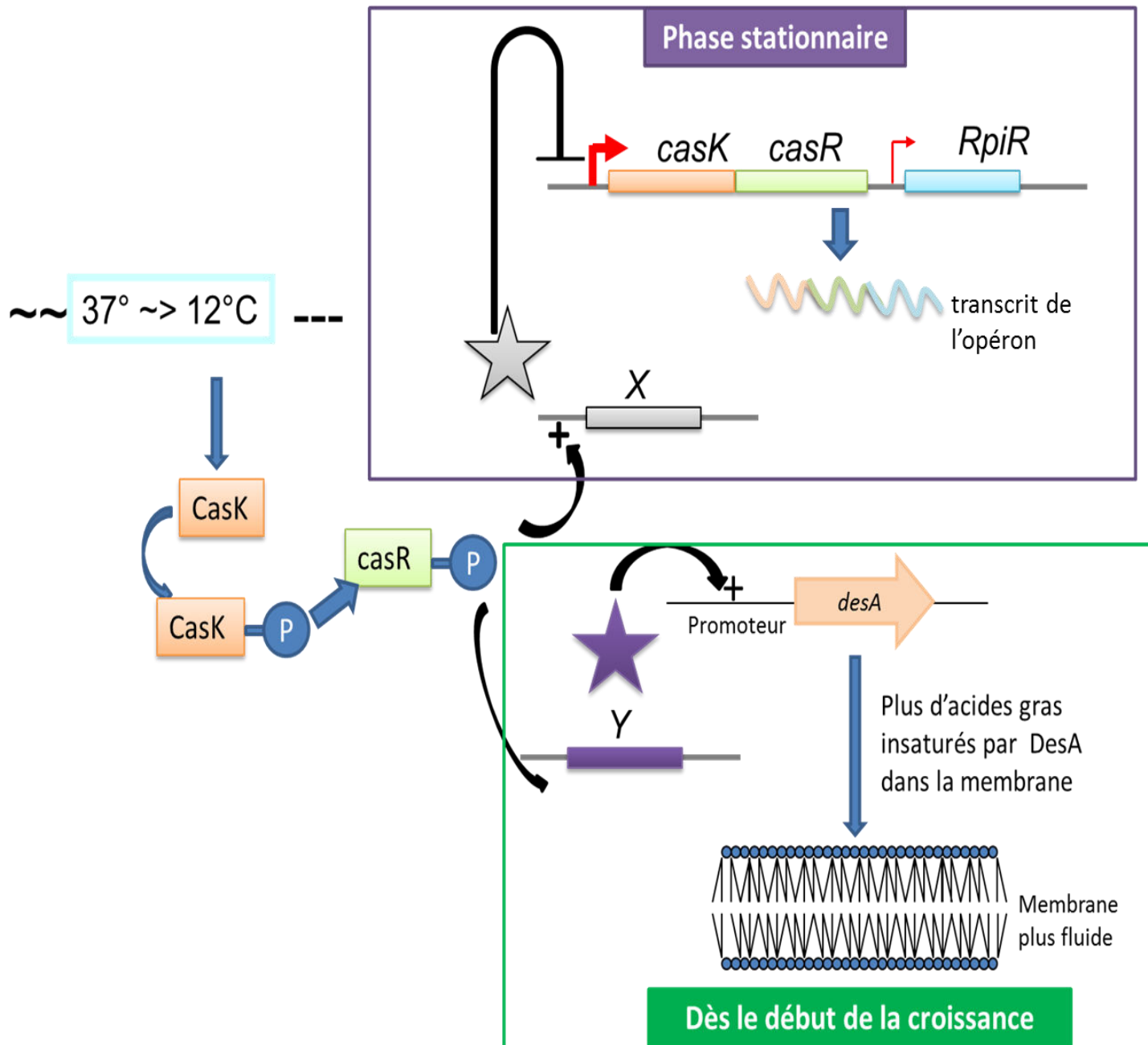


Figure S4 : Fonctionnement de CasK/R à basse température et régulation de l'opéron *casK/R* (phase stationnaire) et du gène *desA* (dès le début de la croissance)

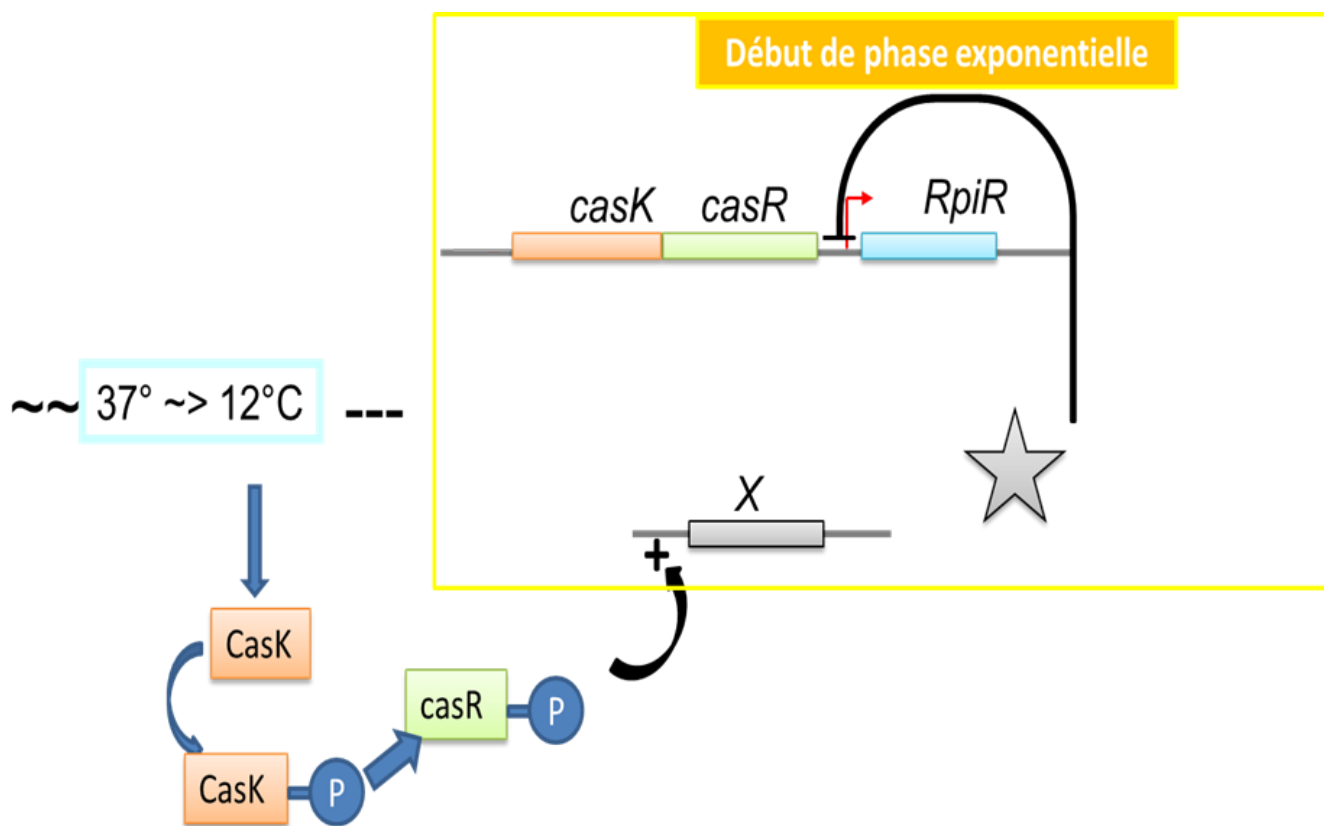


Figure S5 : Fonctionnement de CasK/R à basse température et régulation du gène *rpiR* dès le début de la phase exponentielle.

Même si toutes ces recherches ont apporté de nombreuses informations quant au fonctionnement de CasK/R, des interrogations demeurent néanmoins.

Tout d'abord, il apparaît indispensable de déterminer précisément si la localisation de l'histidine kinase CasK est bien cytoplasmique. En effet, la compréhension des mécanismes de détection du stress froid par CasK en dépend. L'absence de domaine transmembranaire chez une protéine n'exclue pas un ancrage dans la membrane, celle-ci pouvant interagir avec une protéine membranaire qui pourrait être responsable de la perception du signal ou être liée à la membrane par liaisons facilement détachables (Figure S6).

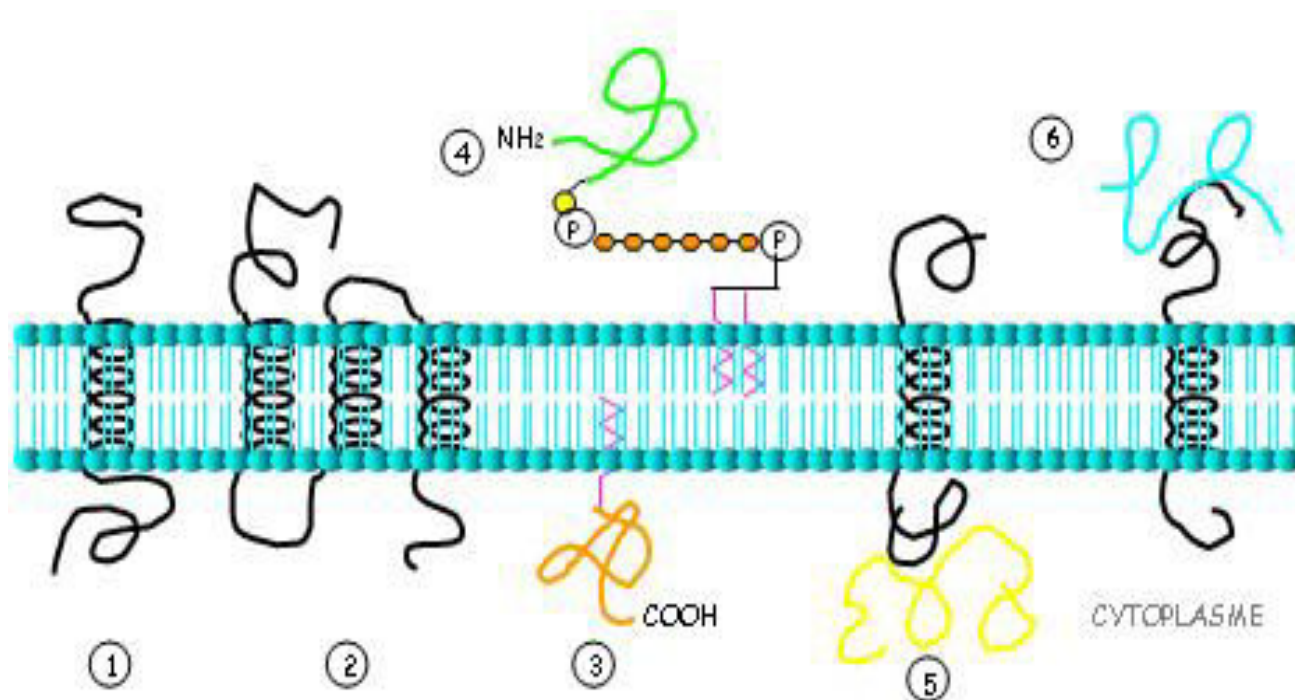


Figure S6 : schéma décrivant les interactions des protéines avec la membrane (Gilbert, 2011). Protéines membranaires intégrales traversant la bicouche lipidique par une (1) ou deux (2) chaîne hélicoïdale d'acides aminés. Protéine (3) liée à un lipide (par un acide gras). Protéine (4) liée par un oligosaccharide à un phospholipide (phosphatidylinositol). Protéines périphériques (5 et 6) liés de façon non covalente à d'autres protéines membranaires

Ainsi pour être sûr de la localisation cytoplasmique de CasK, une analyse structurale de la protéine cristallisée pourrait être réalisée. En plus de cela ou alternativement une cytolocalisation de la protéine CasK pourrait être réalisée, en réalisant par exemple une fusion traductionnelle de l'ORF de CasR avec une protéine fluorescente comme la GFP. Après lyse de la bactérie, l'observation des différentes fractions de lyse (surnageant ou culot) devrait permettre de déterminer la localisation de cette protéine dans la cellule. Le même type d'expérience peut aussi éventuellement être réalisé à l'aide d'un anticorps anti-CasK en présence des différentes fractions cellulaires.

Comprendre comment CasK perçoit le signal de stress « froid » est un point clé dans la compréhension du mécanisme. La présence du domaine GAF comme domaine senseur chez CasK nous permet néanmoins d'avoir des idées sur le type de molécule pouvant jouer le rôle

de molécule signal. Le domaine GAF, sont très présent chez phosphodiésterases régulées par la cGMP. Cependant, d'autres HK avec des domaines GAF sont aussi capables de reconnaître des cGMPc (Ho et al., 2000b). Chez les protéines DOS S et DOS T de *Mycobacterium tuberculosis*, le domaine GAF est capable de se lier à des ligands diatomiques comme O₂, NO et CO (Vos et al., 2012). Toutes ces petites molécules sont souvent décrites dans la littérature comme messagers secondaires jouant un rôle dans la transduction de signaux (Gomelsky, 2011). Généralement, les messagers secondaires sont de petites molécules, qui peuvent donc diffuser rapidement dans le cytoplasme pour permettre au message de circuler rapidement dans la cellule. Le froid ayant un impact sur les voies métaboliques (Graumann and Marahiel, 1996b), il est concevable que le pool cellulaire de ces messagers secondaires soit modifié.

Il pourrait être intéressant de tester, par exemple, l'impact de plusieurs concentrations en divers nucléotides cycliques ou diverses molécules diatomiques sur l'expression du promoteur de l'opéron *casK/R*, sachant que la régulation de ce promoteur est un résultat de l'activation de CasK au froid. A plus long terme, l'étude de l'effet de la mutation du domaine senseur ou du domaine transmetteur de phosphate de CasK permettrait de parfaire les connaissances sur le fonctionnement de cette kinase.

Mieux comprendre le rôle du régulateur RpiR est aussi un point clé qu'il faudrait aborder. Le fait que ce régulateur partage l'opéron avec *casK/R* suggère fortement qu'il serait impliqué dans un même processus biologique à basse température et également à température optimale comme expliqué dans le chapitre 4. Il faudrait donc prévoir la construction d'un mutant, voir un double mutant (avec CasK/R) et voir l'impact de cette (ces) mutation (s) sur l'adaptation au froid de *B. cereus*. Il pourrait être aussi envisageable de purifier tout simplement cette protéine et par retard sur gel voir si ce n'est pas elle qui interagit avec les régions promotrices de *casK/R* et son promoteur individuel, et éventuellement si elle aurait une action sur DesA.

Des expériences ont été réalisées au cours de ma thèse dans ce but mais il s'est avéré difficile d'avoir des clones transformés chez *E. coli*. Les essais réalisés sur une durée de 6 mois, ont par la suite été abandonné au profit d'autres pistes. L'hypothèse de l'éventuelle toxicité de RpiR pour les bactéries était suggérée. Concernant les expériences de mutation du gène *rpiR*, d'autres tentatives pourraient être réalisées dans le futur en modifiant certains paramètres associés au protocole de clonage. Concernant la purification du RpiR, une méthode telle que le « chromosomal his-tagging » pourrait être testée (Mamelli et al., 2007). Il s'agit de réaliser

une construction contenant le gène *rpiR* avec une étiquette His-tag en Cter cloné dans un vecteur suicide possédant une résistance à un antibiotique, puis introduire ce plasmide chez *B. cereus* et intégrer par simple recombinaison homologue la construction au chromosome de la bactérie. L'expression du gène devrait être donc sous le contrôle de son propre promoteur.

Face au nombre de données générés par le transcriptome déterminer tous les partenaires potentiels de CasK/R s'est avéré assez délicat. Ainsi une étude supplémentaire s'avèrerait indispensable. Pour cela, des expériences de co-immunoprécipitation avec l'histidine CasK lors d'une croissance au froid suivie d'une analyse en spectromètre de masse pourraient être réalisées. En effet, les systèmes de transductions de signaux peuvent être des réseaux complexes impliquant parfois plusieurs systèmes à deux composants, plusieurs régulateurs, mais aussi des phosphorylations croisée entre 2 systèmes à deux composants (Siryaporn and Goulian, 2008, Podgornaia and Laub, 2013a, Kenney, 2010, Boll and Hendrixson, 2011, Mascher, Jung et al., 2012).

Une alternative à cette méthode serait la méthode du double hybride. En effet dans le chapitre 5, une liste de régulateurs potentiellement régulés par CasK/R a été mise en évidence. Ils pourraient justement être testés comme partenaires de CasK ou CasR.

Il serait intéressant de révéler les différentes régions promotrices régulées par le régulateur CasR et ce avec une approche sans-à-priori telle que le ChIP-sequencing, qui combine la méthode du ChiP (chromatin immunoprecipitation) avec du séquençage d'ADN pour identifier les sites de fixations de la protéine d'intérêt. Sinon, il pourrait être envisageable de vérifier par EMSA la fixation de CasR sur les promoteurs des gènes de régulateurs identifiés dans l'analyse transcriptomique (Chapitre 5).

Des gènes codant pour des protéines liées à la spore ou à la sporulation ont été identifiés lors de l'analyse transcriptionnelle (Chapitre 5). Il serait donc pertinent de déterminer si la mutation de *casK/R* a réellement un impact sur les capacités de sporulation et/ou de germination des spores à basse température.

D'autres perspectives peuvent être envisageables à plus long terme : les souches thermotolérantes du groupe 7 étant dépourvues des gènes *casK/R* (chapitre 1), on pourrait tout

à fait envisager de voir l'impact de l'ajout de copie de *casK/R en trans* sur la capacité de croissance de ces souches au froid. Une telle approche a d'autant plus d'intérêt que toutes les souches du groupe 7 appartiennent à une même espèce, *B. cytotoxicus* très souvent isolée de toxi-infection alimentaire collective (TIAC). L'acquisition de gènes d'adaptation au froid par ses souches pourrait conduire à une augmentation des cas TIAC répertoriés. L'impact de la présence de *casK/R* sur la cytotoxicité de cette espèce pourrait aussi être étudié. Toutes ces données permettraient d'améliorer les modèles de prédictions du comportement de ces souches pathogènes dans l'aliment.

Il serait possible d'avoir à peu près le même type d'approche mais cette fois sur des souches (autre que celle de *B. cereus sl*) d'intérêt technologique, alimentaire ou médicale. Il s'agirait plus précisément de vérifier si rajouter *en trans casK/R* chez ces souches permettrait d'améliorer leur capacité à persister lors de procédés de stockages tels que la réfrigération, non envisageables pour certaines d'entre elles.

La présence de *casK/R* dans un grand nombre de souches du groupe *B. cereus sensu lato* permet d'envisager de proposer ces gènes comme marqueurs pour l'identification de souches pathogènes mésophiles ou psychrotolérantes du groupe *B. cereus* isolées d'aliments réfrigérés.

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Valorisation des travaux de thèse

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Communications scientifiques

2014

• **Présentation orale** : Adaptation au froid chez *Bacillus cereus sensu lato*: Implication d'un senseur/régulateur. Sara Esther Diomandé, Christophe Nguyen-the, Tjakko Abee, Marcel Tempelaars, Véronique Broussolle and Julien Brillard. Carry-le-rouet, 6-10 Octobre 2014

• **Présentation orale**: A novel mechanism involved in *Bacillus cereus* cold adaptation. Sara Esther Diomandé, Christophe Nguyen-the, Tjakko Abee, Marcel Tempelaars, Véronique Broussolle and Julien Brillard. Nantes, 1^{er} au 4 Septembre 2014. **1^{er} prix de la communication orale**

• **Présentation orale** : Implication du système à deux composants CasK/R dans l'adaptation de *Bacillus cereus sensu lato* au froid. Sara Esther Diomandé, Christophe Nguyen-the, Tjakko Abee, Marcel Tempelaars, Véronique Broussolle et Julien Brillard. Séminaire centre INRA Avignon, 19 Juin 2014, Avignon.

2013

• **Présentation orale** : Rôle du système senseur/régulateur CasKR dans l'adaptation au froid de la bactérie pathogène *Bacillus cereus*. Sara Diomandé, V. Broussolle, J. Brillard. Journée des doctorants de l'UMR408. 17 déc. 2013, Avignon.

• **Présentation affichée** : Adaptation au froid chez *Bacillus cereus* : Implication d'un senseur/régulateur. Sara Diomandé, V. Broussolle, J. Brillard. 9e Congrès National de la Société Française de Microbiologie. Lille - 7 et 8 février 2013

2012

• **Présentation orale** : Rôle d'un système senseur/régulateur dans l'adaptation au froid de la bactérie pathogène *Bacillus cereus*. Sara Diomandé, V. Broussolle, J. Brillard. Journée des doctorants de l'UMR408. 17 déc. 2012, Avignon.

• **Présentation affichée** : Adaptation de *Bacillus cereus* au froid : mécanismes moléculaires impliqués et exploitation de la diversité génétique du groupe *Bacillus cereus*. Sara Diomandé, V. Broussolle, J. Brillard. Journées des microbiologistes de l'Inra 2012. 13-15 Nov. 2012. L'Isle-sur-la-Sorgue (84).

• **Présentation orale** : Adaptation au froid de la bactérie pathogène *Bacillus cereus*. Sara Diomandé, V. Broussolle, J. Brillard. Rencontre avec les responsables qualité de l'entreprise Provence Plats (partenaire socio-économique de la thèse), 6 Juillet 2012, Rognonas.

2011

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Publications scientifiques

Article publié

- Diomandé SE, Chamot S, Antolinos V, Vasai F, Guinebretière MH, Bornard I, Nguyen-the C, Broussolle V, Brillard J (2014) . **The CasKR two-component system is required for the growth of mesophilic and psychrotolerant *Bacillus cereus* strains at low temperatures.**

Articles soumis ou en préparation

- Sara Esther Diomandé, Christophe Nguyen-the, Tjakko Abee, Marcel H. Tempelaars, Véronique Broussolle and Julien Brillard. **Involvement of CasK/R Two-component system in optimal unsaturation of the *Bacillus cereus* fatty acids during low-temperature growth.** Soumission pour publication dans International Journal of Food microbiology
- Sara Esther Diomandé, Bénédicte Doublet, Florian Vasai, Véronique Broussolle and Julien Brillard. **Expression et régulation originales du système à deux composants CasK/R chez *Bacillus cereus*.** Soumission prévue dans le journal FEMS microbiology letters
- Sara Esther Diomandé, Christophe Nguyen-the, Marie-Hélène Guinebretière, Véronique Broussolle and Julien Brillard. **Role of fatty acids in *Bacillus* strains adaptation to environment.** Soumission prévue dans le journal Frontiers in Microbiology

ADAPTATION DE *BACILLUS CEREUS* AU FROID : MECANISMES MOLECULAIRES IMPLIQUES ET EXPLOITATION DE LA DIVERSITE GENETIQUE DU GROUPE *BACILLUS CEREUS*

AUTEURS : SARA ESTHER DIOMANDE^{1,2}, VERONIQUE BROUSSOLE^{1,2}, JULIEN BRILLARD^{1,2}.

EMAIL : sara-esther.diomande@avignon.inra.fr

¹ INRA, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France

² Université d'Avignon et des Pays de Vaucluse, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France.

Bacillus cereus, bacille ubiquitaire, est un contaminant fréquent des aliments. Sa capacité à sporuler lui permet de survivre lors de la transformation des aliments. Il est également capable de s'adapter à différents types d'environnements. *B. cereus sensu lato* est divisé en sept groupes phylogénétiques qui peuvent être catégorisés en trois thermotypes : thermotolérants, mésophiles et psychrotrophes[1].

B. cereus représente le 4^e agent responsable de TIAC (Toxi-Infection Alimentaire Collective) en France, mais les doses infectieuses ($>5.10^4$ UFC) nécessitent qu'une prolifération ait eu lieu dans l'aliment avant ingestion par le consommateur. Les souches psychrotrophes, potentiellement capables de croître durant la conservation des aliments à basses températures représentent ainsi un risque de TIA.

Le système à deux composants YhcY/Z est un système senseur/régulateur homologue au système Des K/R décrit chez *Bacillus subtilis* pour son rôle dans l'adaptation de cette souche au froid[2]. La caractérisation de ce système majoritairement conservé chez *B. cereus sensu lato* est en cours. Nos résultats indiquent qu'il est surexprimé aux basses températures chez *Bacillus cereus* ATCC 14579. De plus, des mutants de ce système, construits chez deux souches de groupes phylogénétiques différents, sont affectés dans leur croissance et leur survie aux basses températures.

1. Guinebretière, M.-H., et al., *Ecological diversification in the Bacillus cereus Group*. Environmental Microbiology, 2008. **10**(4): p. 851-865.
2. Cybulski, L.E., et al., *Bacillus subtilis DesR Functions as a Phosphorylation-activated Switch to Control Membrane Lipid Fluidity*. Journal of Biological Chemistry, 2004. **279**(38): p. 39340-39347.



Cold adaptation of *Bacillus cereus*: molecular mechanisms involved and exploitation of the *Bacillus cereus sensu lato* genetic diversity



SARA ESTHER DIOMANDE*, VERONIQUE BROUSSOLLE, JULIEN BRILLARD.
INRA, UMR4085 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France.
*SARA DIOMANDE (sara-esther.diomande@avignon.inra.fr). Thesis funded by INRA MICA and Région PACA

INTRODUCTION

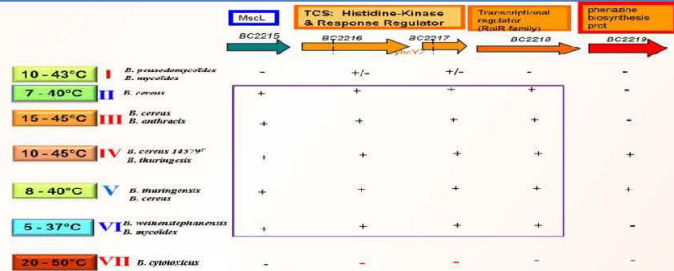
Bacillus cereus is a foodborne pathogen with a high ability of adaptation leading to resistance to food transformation processes and conservation. The *Bacillus cereus sensu lato* is divided into 7 genetic groups which are correlated to the ability to grow at a given range of temperature. Thus, 7 « thermotypes » have been defined. They include psychrotolerant, mesophilic, and thermotolerant strains [1]. Identifying the mechanisms involved in *B. cereus* cold adaptation may help in understanding how psychrotolerant strains are able to grow in food during refrigeration. Our purpose is to investigate the implication of a two component system (TCS) in cold adaptation of two *B. cereus* strains ATCC 14579 (phylogenetic group IV) and AH 187 (phylogenetic group III) and to characterize the mechanisms involved in this adaptation.

Presentation of the TCS

Two component systems are composed of a sensor protein and a regulator protein.

The TCS locus

In ATCC 14579, belonging to the phylogenetic group IV, the TCS is encoded by the genes *BC2216-17*. In silico analysis of these TCS genes among *Bacillus cereus sensu lato* strains showed that the synteny of the locus is conserved among strains of group II – III – IV – V and VI.



RESULTS

Growth at low temperature

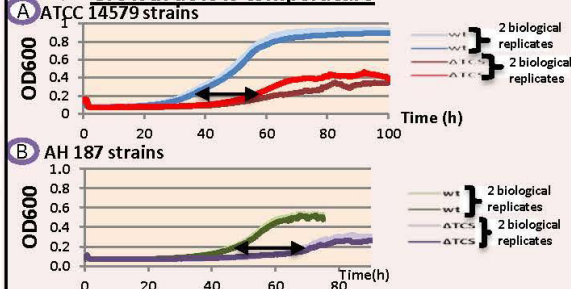


Figure 1: Growth curves of *B. cereus* ATCC 14579 (A), AH187 (B) strains and their respective mutants at 12°C.

Isogenic mutants of ATCC 14579 (*bc2216-2217*) and AH 187 (*bcAH187_A2374 & A2375*) were obtained by insertion of a tetracycline resistance cassette interrupting the TCS genes. The growth was performed in LB medium in an automatic turbidimeter.

For the two wild type (wt) strains, a shorter lag phase was observed compared to the mutant strains (ΔTCS) as well as a higher maximal OD.

No phenotypic difference was observed during acid, basic, oxidative or high temperature stresses between ATCC 14579 wt and ΔTCS strains (data not shown).

These results suggest that the TCS is necessary for the growth of *B. cereus* group III and group IV strains at low temperature.

Survival at 4°C

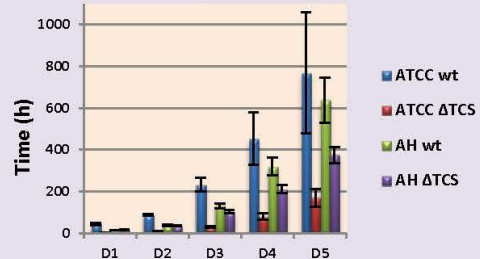


Figure 2: Survival at 4°C in *B. cereus* ATCC 14579 and AH187 strains and their respective mutants.

Bacterial suspensions at 10^6 CFU/ml were incubated at 4°C and the CFU were enumerated over time. D1, D2, D3, D4, D5 represent the decrease of 1, 2, 3, 4, 5 log of bacterial population for the (wt) and the mutant (ΔTCS) of each strain. The ATCC 14579 wt had a longer time of population decrease than the mutant from D1 to D5. In AH187, this difference appeared only for D3 and above.

Moreover, no difference in survival time was observed between the two wt strains. So, the survival at 4°C was not dependent on their thermotype.

No difference was observed for survival at 37°C (data not shown) suggesting that the TCS is involved in the cold survival of these strains.

Expression of the TCS in ATCC14579

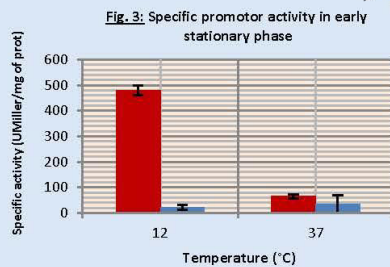


Fig. 3: Specific promoter activity in early stationary phase

A transcriptional fusion was performed by cloning the promoter region of the TCS gene (*bc2216*) in a plasmid containing the *lacZ* gene. ATCC 14579 WT and the TCS mutant were transformed, and β-galactosidase activity measurement was done during growth at 12 and 37°C.

The TCS promoter activity displayed a basal expression (about 10 Miller Units) in these strains at 12°C and at 37°C during exponential phase (data not shown), then increased in early stationary phase (Fig. 3).

A higher TCS promoter activity was observed at 12°C compared to that observed at 37°C in the mutant strain (Δ16). No significant difference was observed in the wild type (W15). The mutation of the TCS caused an overexpression (or a de-repression) of the TCS promoter activity. So, the TCS seems to autoregulate its promoter activity, either directly or not.

CONCLUSION/PERSPECTIVES

The studied two component system plays a key role in cold adaptation and cold survival of those 2 strains belonging to different thermotypes. The molecular mechanism involved in this adaptability is currently studied. The construction of TCS mutants in psychrotolerant strains is underway in order to determine if its role in cold adaptation could be extended to *Bacillus cereus sensu lato*.

1. Guinebretiere M.H., et al., Ecological diversification in the *Bacillus cereus* Group. Environmental Microbiology, 2008, 10(4): p. 651-665

10^e congrès de la Société Française de Microbiologie 2013, Lille, France (Poster)

Adaptation au froid chez *Bacillus cereus* : Implication d'un senseur/régulateur

AUTEURS : SARA ESTHER DIOMANDE^{1,2}, VERONIQUE BROUSSELE^{1,2}, JULIEN BRILLARD^{1,2}.

EMAIL : sara-esther.diomande@avignon.inra.fr

¹ INRA, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France

² Université d'Avignon et des Pays de Vaucluse, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France.

Le but de cette étude est d'évaluer le rôle d'un senseur/régulateur dans l'adaptation au froid de souches de *Bacillus cereus sensu lato* de thermotypes différents.

B. cereus sensu lato est constitué de plusieurs espèces ubiquitaires parmi lesquelles des pathogènes connus comme *B. anthracis*, *B. thuringensis* et *B. cereus sensu stricto*. Ces bactéries capables de s'adapter à divers environnements peuvent aisément résister aux conditions rencontrées lors de la transformation et de la réfrigération des aliments. Les souches de *B. cereus sensu lato* sont réparties en 7 groupes phylogénétiques caractérisés par différentes capacités à se développer au froid ou au chaud, correspondant ainsi à des "thermotypes" différents. Les souches psychrotolérantes sont considérées comme des pathogènes émergents. Peu d'études décrivent les mécanismes impliqués dans l'adaptation au froid de *B. cereus*. Nous avons mis en évidence un système senseur/régulateur surexprimé au froid, très conservé chez les souches de *B. cereus sensu lato* y compris chez les souches psychrotolérantes.

Deux souches de thermotypes et de groupes phylogénétiques différents ont été étudiées, ainsi que leur mutants isogéniques respectifs possédant une version tronquée du senseur/régulateur. Des études de physiologie bactérienne ont été réalisées sur ces souches, à de basses températures favorables ou non à leur croissance. Les données obtenues ont été comparées à celles observées aux conditions optimales de croissance des deux souches (37°C), et lorsqu'elles sont soumises à différents autres stress.

Les mutants du senseur/régulateur présentent un retard, voire une absence de croissance aux basses températures par rapport aux sauvages. Lorsque des retards de croissance sont observés, aucune différence entre les taux de croissance n'est visible entre les souches, suggérant l'implication du senseur/régulateur durant la phase de latence. A des températures basses non favorables à la croissance de ces bactéries, une meilleure survie des souches sauvages par rapport aux mutants est observée. Néanmoins, aucune différence n'est notée entre les souches à 37°C et lorsqu'elles sont soumises à d'autres stress. D'autre part, les profils d'acides gras obtenus chez ces souches sont similaires aux basses températures comme à 37°C.

Ces résultats montrent que le senseur/régulateur joue un rôle important dans l'adaptation au froid chez les souches étudiées. Des études sont en cours de réalisation chez des espèces psychrotolérantes afin d'étendre nos conclusions à la diversité de souches que compte *B. cereus sensu lato*.



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Adaptation au froid chez *Bacillus cereus* : Implication d'un senseur/régulateur



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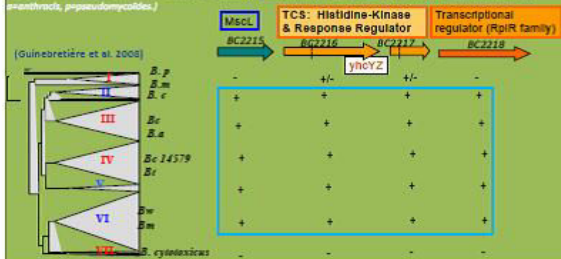
SARA ESTHER DIOMANDE*, VERONIQUE BROUSSOLLE, JULIEN BRILLARD.
INRA, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France.
*SARA DIOMANDE [sara-esther.diomande@avignon.inra.fr] - Thesis funded by INRA MICA and Région PACA

B. cereus sensu lato est constitué de plusieurs espèces ubiquitaires parmi lesquelles des pathogènes connus comme *B. anthracis*, *B. thuringiensis* et *B. cereus sensu stricto*. Ces bactéries capables de s'adapter à divers environnements peuvent aisément résister aux conditions rencontrées lors de la transformation et de la réfrigération des aliments. Les souches de *B. cereus sensu lato* sont réparties en 7 groupes phylogénétiques caractérisés par différentes gammes de températures de croissance. Les souches psychrotolérantes sont considérées comme des pathogènes émergents. Peu d'études décrivent les mécanismes impliqués dans l'adaptation au froid de *B. cereus*. Nous avons mis en évidence un système à deux composants (TCS) ou senseur/régulateur, surexprimé au froid, très conservé chez les souches de *B. cereus sensu lato* y compris chez les souches psychrotolérantes. Le but de cette étude est d'évaluer le rôle de ce TCS dans l'adaptation au froid de souches de *Bacillus cereus sensu lato* de thermotypes différents.

Bacillus cereus sensu lato et le TCS YhcY/Z
Tableau listant les 7 groupes phylogénétiques de *Bacillus cereus sensu lato* (Guinebretière et al., 2008)

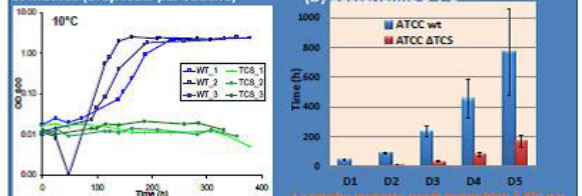
| Groupes phylogénétiques | Gamme de température de croissance (°C) | Thermotypes |
|-------------------------|---|-----------------|
| Groupe I | 10-43 | Mésophile |
| Groupe II | 7-40 | Psychrotolérant |
| Groupe III | 15-45 | Mésophile |
| Groupe IV | 10-45 | Mésophile |
| Groupe V | 8-40 | Mésophile |
| Groupe VI | 5-37 | Psychrotolérant |
| Groupe VII | 20-50 | Thermotolérant |

La synthèse des gènes du TCS est conservée chez les souches de *Bacillus cereus sensu lato* sauf celles des 2 groupes les plus éloignés des autres, le groupe I et le groupe VII (thermotolérant) (*B. bacillus*, *B. myosotidis*, *B. thuringiensis*, *B. cereus*, *B. weihenstephanensis*, *B. anthracis*, *B. pseudomycoides*).



Adaptation au froid de la souche modèle ATCC 14579 (groupe IV)

Croissance à 10°C des souches sauvages et mutées (3réplicats par souche) | Temps de décroissance de population (D) bactérienne à 4°C



La souche mutante Δ TCS est affectée pour sa croissance à basse température.

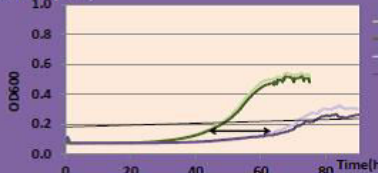
Vérification de la complémentation de la souche mutante par observation de croissance à 10°C de différentes concentrations des souches bactériennes



La complémentation de la souche mutante par les gènes du TCS permet la croissance à 10°C suggérant que la capacité de croissance à cette température nécessite la présence du TCS.

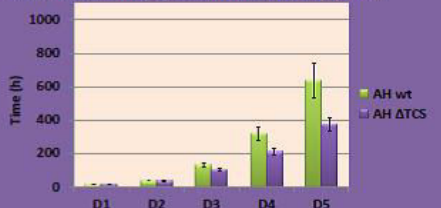
Adaptation au froid chez la AH187 (groupe III)

Croissance au froid (12°C)



Chez la souche AH187 (un groupe plus mésophile que celui de l' ATCC14579), un retard de croissance est observé chez la souche mutante à 12°C par rapport à la souche sauvage suggérant l'implication du TCS dans l'adaptation au froid.

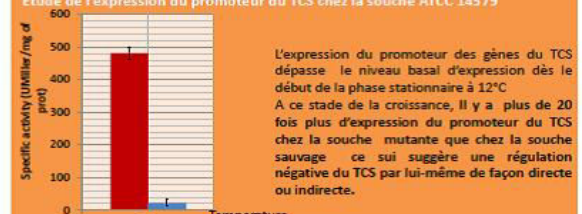
Temps de décroissance de population bactérienne (D) à 4°C



Comme chez la souche ATCC 14579, la souche AH187, mutante pour le TCS, survit moins bien à 4°C par rapport à la souche sauvage car le temps nécessaire pour une même décroissance est plus faible que pour la souche sauvage

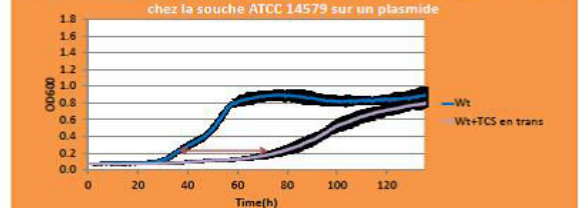
Des pistes sur la régulation du TCS chez la souche ATCC 14579

Etude de l'expression du promoteur du TCS chez la souche ATCC 14579



L'expression du promoteur des gènes du TCS dépasse le niveau basal d'expression dès le début de la phase stationnaire à 12°C. A ce stade de la croissance, il y a plus de 20 fois plus d'expression du promoteur du TCS chez la souche mutante que chez la souche sauvage ce qui suggère une régulation négative du TCS par lui-même de façon directe ou indirecte.

Surexpression du TCS par introduction d'une copie supplémentaire des gènes chez la souche ATCC 14579 sur un plasmide



L'introduction d'une copie supplémentaire du TCS conduit à une diminution de la capacité de croissance au froid à 12°C, une perte d'adaptabilité suggérant la nécessité d'une régulation fine de l'expression du TCS chez la souche ATCC 14579 sauvage.

- Ces résultats mettent en évidence que :
 - le TCS YhcY/Z joue un rôle important dans l'adaptation et la survie au froid de deux souches de groupes phylogénétiques différents de *Bacillus cereus sensu lato*
 - l'expression des gènes de ce TCS semble finement régulée chez la souche ATCC 14579.
- Perspectives :
 - étudier le TCS chez des espèces psychrotolérantes de *Bacillus sensu lato*
 - comprendre comment se fait la perception du signal
 - mettre en évidence le rôle du TCS pour l'adaptation au froid.



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Adaptation au froid chez *Bacillus cereus sensu lato*: Implication d'un senseur/régulateur

Sara Esther Diomandé^{1,2}, Christophe Nguyen-the^{1,2}, Tjakko Abée^{3,4}, Marcel Tempelaars⁴,
Véronique Broussolle^{1,2} and Julien Brillard^{1,2}.

¹ INRA, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France

² Université d'Avignon, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France.

³ Top Institute Food and Nutrition, NieuweKanaal 9A, 6709 PA, Wageningen, The Netherlands

⁴ Food Microbiology Laboratory, Wageningen University, BornseWeilanden 9, 6708 WG, Wageningen, The Netherlands

Abstract

Bacillus cereus sensu lato (*sl*) est constitué de 7 espèces ubiquitaires génétiquement très proches incluant des pathogènes connus comme *B. anthracis*, *B. thuringensis* et *B. cereus sensu stricto*. Elles sont réparties en 7 groupes phylogénétiques présentant des gammes de température de croissance variées. *Bacillus cereus sl* est ainsi caractérisé par 3 thermotypes principaux: thermotolérants, mésophiles, psychrotolérants.

Nous avons mis en évidence que le système senseur/régulateur CasK/R, conservé chez les souches de *B. cereus sl* excepté parmi les souches thermolérantes, est surexprimé durant la croissance à basse température. Quatre souches de thermotypes différents, ainsi que leurs mutants isogéniques de *casK/R* respectifs ont été étudiés [1]. Les croissances à basses températures ont été comparées à celles réalisées aux conditions optimales de croissance (37°C). Les 4 souches mutantes de *casK/R* présentent un retard, voire une absence de croissance à basses températures par rapport aux souches sauvages mais aucune différence n'est observée à 37°C ou en présence d'autres stress. Ces résultats montrent clairement que CasK/R est un mécanisme générique d'adaptation de *B. cereus sl* au froid, quel que soit le thermotype.

Une étude transcriptomique réalisée sur une souche modèle et son mutant isogénique $\Delta casK/R$ a révélé une centaine de gènes différentiellement exprimés en début de phase exponentielle et en phase stationnaire, à basse température. Douze de ces gènes, pour la plupart down-régulés, codent pour des protéines impliquées dans le métabolisme des acides gras, incluant les gènes *desA* et *desB* codant pour les 2 désaturases de *B. cereus*. Les profils d'acides gras (AG) des membranes des 2 souches, obtenus par GC/MS, indiquent une plus faible proportion d'AG insaturés par DesA chez la souche $\Delta casK/R$. Aucune différence entre les 2 souches n'est observée pour les AG insaturés par DesB. Le dosage de l'activité du promoteur du gène *desA* chez les souches WT et $\Delta casK/R$ a permis de mettre en évidence une sous-expression du promoteur de ce gène chez $\Delta casK/R$, durant la croissance au froid. CasK/R régulerait donc l'expression de *desA* lors de la croissance de *B. cereus* à basse température. Cette étude permet de mieux comprendre par quels mécanismes *B. cereus sl* s'adapte au froid, rencontré lors de la conservation des produits alimentaires.

Référence

1. Diomandé, S., et al., *The CasKR two-component system is required for growth at low temperature of mesophilic and psychrotolerant Bacillus cereus strains*. Applied and Environmental Microbiology, 2014 ; 80: 2493-503.

A novel mechanism involved in *Bacillus cereus* cold adaptation

Sara Esther Diomandé^{1,2}, Christophe Nguyen-the^{1,2}, Tjakko Abee^{3,4}, Marcel Tempelaars⁴,
Véronique Broussolle^{1,2} and Julien Brillard^{1,2}.

¹ INRA, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France

² Université d'Avignon, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France.

³ Top Institute Food and Nutrition, NieuweKanaal 9A, 6709 PA, Wageningen, The Netherlands

⁴ Food Microbiology Laboratory, Wageningen University, BornseWeilanden 9, 6708 WG, Wageningen, The Netherlands

Corresponding author: SARA DIOMANDE : sara-esther.diomande@avignon.inra.fr

Bacillus cereus sensu lato comprises ubiquitous strains consisting of thermotolerant, mesophilic and psychrotolerant strains. Psychrotolerant and some mesophilic strains, identified as emerging human pathogens, can survive to the conditions encountered during food processing and grow in refrigerated food. The mechanisms involved in *B.cereus* cold adaptation are scarcely known. We identified a new sensor/regulator system named CasK/R, conserved among strains of *B.cereus sl*, except in thermotolerant strains, that plays a key role in cold adaptation.

Growth of four isogenic $\Delta casK/R$ mutants belonging to mesophilic and psychrotolerant groups was carried out at low temperature and at optimal growth temperature. The four $\Delta casK/R$ mutants tested were all impaired in their ability to adapt to low temperature, compared to the parental strains, but no difference was observed at the optimal growth temperature. This suggests that CasK/R is a generic mechanism involved in *B.cereus sl* cold adaptation.

Transcriptomic analysis performed with the ATCC14579 strain showed more than 100 genes differentially expressed in the $\Delta casK/R$ mutant *versus* parental strain, during growth at 12°C. Interestingly, the two desaturases DesA and DesB were down-regulated in the mutant. Fatty acid (FA) profiles analysed with GC/MS indicated a lower proportion of FAs presumably unsaturated by DesA in the $\Delta casK/R$ strain compared to the parental strain. No difference was found for FAs presumably unsaturated by DesB. When the medium was supplemented with unsaturated FAs, $\Delta casK/R$ was able to grow similarly as the parental strain at low temperature, indicating that exogenous unsaturated FAs can compensate for membrane FAs composition modifications caused by the lack of CasKR during *B.cereus* growth at low temperature.

This work may help predict how *B.cereus* behaves in refrigerate food and *in fine* more accurately estimate the risk of *B.cereus* proliferation in chilled food.

Annexes

Annexes Chapitre 2

Table X. Growth of WT and $\Delta bc2216-17$ mutant in various stressful conditions

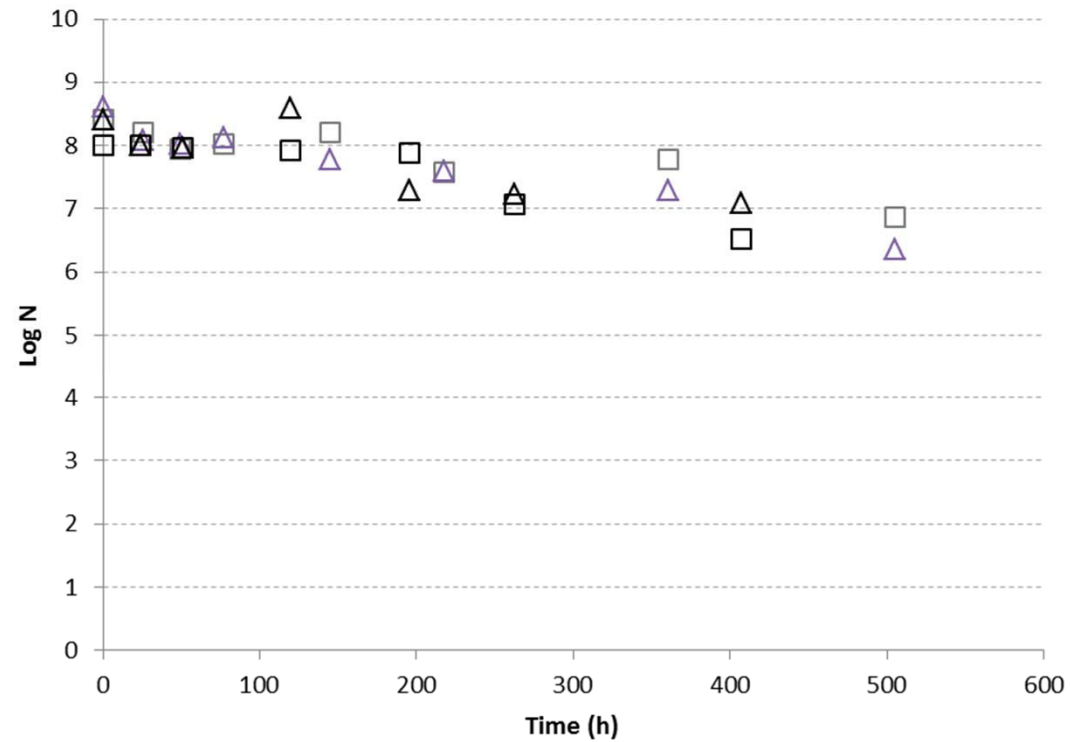
| Stress tested | Stress condition | Growth condition | Time to reach stationary phase for both strains | Conclusion for the 2 strains |
|------------------------------|--------------------------------------|------------------|---|------------------------------|
| high temperature | 42°C | microplate | OD>1 in 3.5h | similar growth |
| | 45°C | microplate | OD>1 in 4.5h | similar growth |
| acid (HCl supplementation) | pH 5.5 | microplate | OD>1 in 7h | similar growth |
| | pH 5.0 | microplate | OD>1 in 12h | similar growth |
| | pH 4.7 | microplate | OD>1 in 32h | similar growth |
| basic (NaOH supplementation) | pH 8.5 | microplate | OD>1 in 12h | similar growth |
| | pH 8.6 | microplate | OD>1 in 13h | similar growth |
| Oxydative agent | 100µM paraquat | microplate | OD>1.5 in 16h | similar growth |
| | 150µM paraquat | microplate | OD>1.3 in 14h | similar growth |
| | AAPH 100mM | microplate | OD>1.2 in 12h | similar growth |
| | AAPH 150mM | microplate | OD>1.0 in 12h | similar growth |
| | 0.020% H ₂ O ₂ | microplate | OD>1 in 12h | similar growth |
| | 0.025% H ₂ O ₂ | microplate | OD>1 in 12h | similar growth |
| high osmolarity | 4 % NaCl | microplate | OD>1 in 18h | similar growth |
| | 6 % NaCl | microplate | OD>0.8 in 36h | similar growth |
| low osmolarity | 0 % NaCl | flask | OD>1 in 4h | similar growth |
| Ethanol | 4% | flask | OD>1 in 3h | similar growth |
| | 6% | flask | OD>0.1 in 9h | similar growth |
| Ions chelators | EDTA 0.1mM | flask | OD>1 in 7h | similar growth |
| | EGTA 0.1mM | flask | OD>0.5 in 8h | similar growth |
| | 2,2'-bipyridyl 100µM | flask | OD>0.2 in 5h | similar growth |
| | 2,2'-bipyridyl 500µM | flask | OD>0.1 in 5h | similar growth |

Growth in Erlenmeyer flasks was performed in 50ml LB at 37°C with shaking at 200rpm, after inoculation to an OD of 0.01 by an overnight grown culture. OD was measured in microcubes. Growth in microplates was performed in 250 µl LB at 37°C with shaking, after inoculation by 10µl of an overnight grown culture in an automatic turbidimeter (see material & methods section). OD was measured every 15 min.

Temperature was set at 37°C, pH at 7, and NaCl concentration at 1%, unless stated differently.

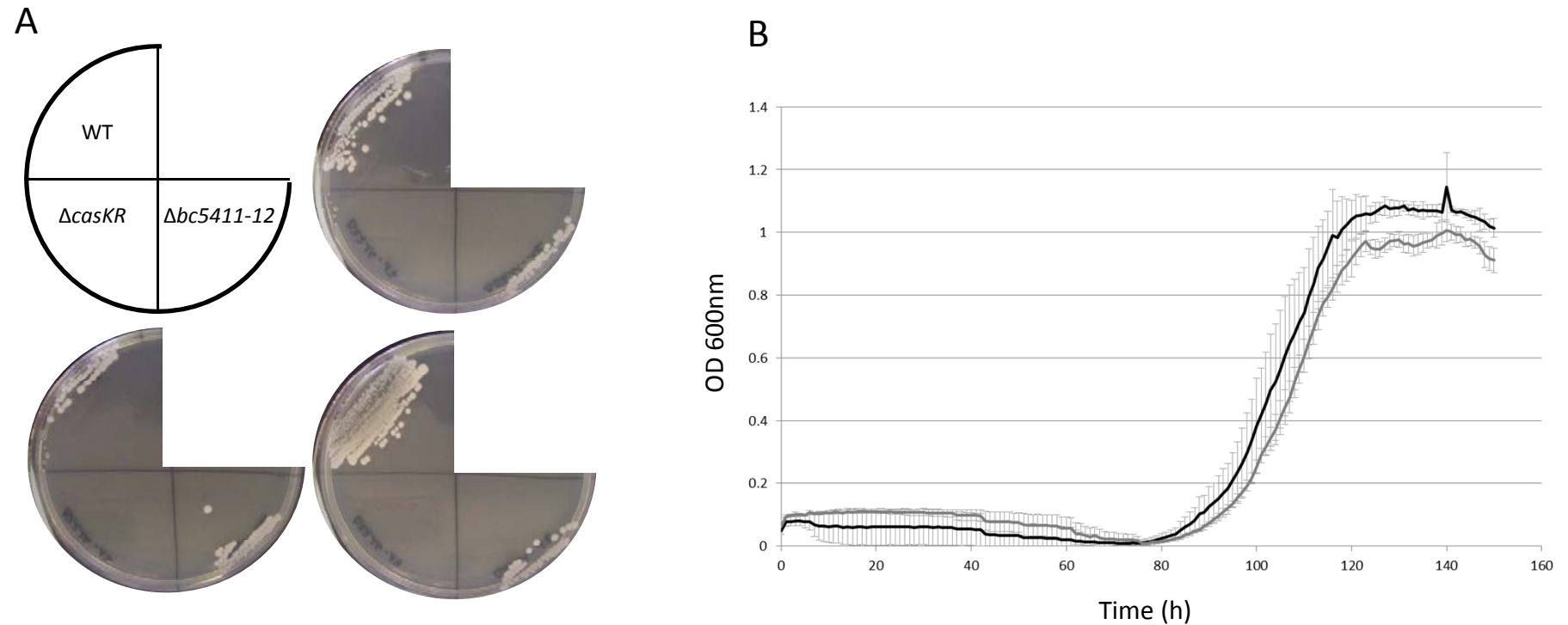
Supp. data S2

Survival of *B. cereus* WT and $\Delta casKR$ strains at 37°C



Bc WT (squares) and $\Delta casKR$ (triangles) bacterial suspension were incubated in LB at 37°C. Cfu numeration was performed every day. Black and grey represent data from replicates 1 and 2, respectively.

Supp. data S3. The $\Delta bc5411-12$ mutant can grow at low temperature



A. The $\Delta bc5411-12$ mutant can grow at T_{min} . One colony of overnight grown cultures of *B. cereus* WT, $\Delta bc5411-12$ and $\Delta casKR$ were streaked on LB agar and incubated at 10°C for 14 days. Three independent replicates are shown.

B. The $\Delta bc5411-12$ mutant can grow at 12°C , similarly as the WT strain. Growth of WT (black) and $\Delta bc5411-12$ (grey) strains was performed in an automated turbidimeter with shaking at 12°C . Mean values \pm SD of three biological replicates are shown.

Les annexes S4 et S5 sont disponibles en ligne à l'adresse suivante :

<http://aem.asm.org/content/80/8/2493.long>

Annexes chapitre 2

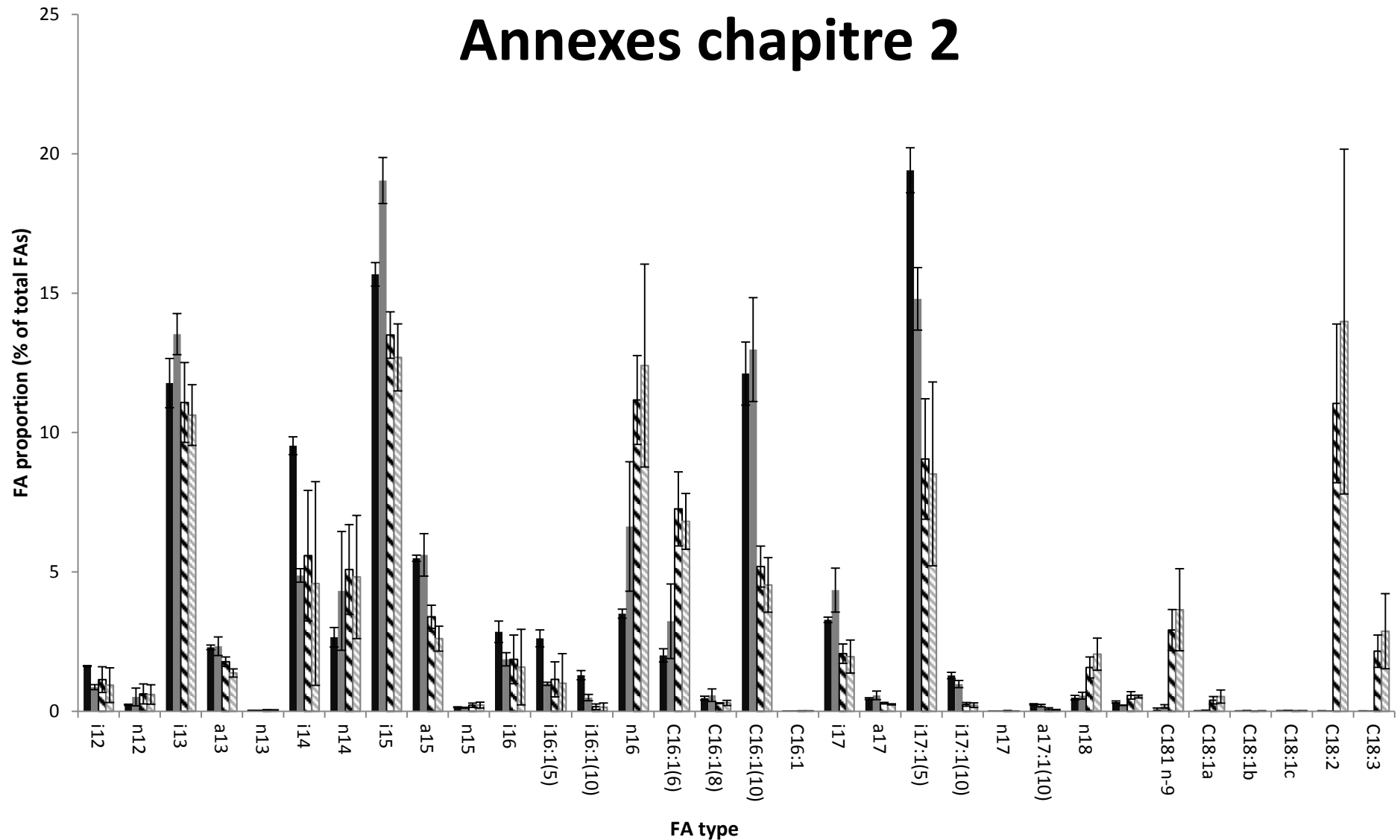


Figure S1: Figure representing WT and $\Delta casK/R$ FA composition in percent of total fatty acids. The fatty acids were extracted after a culture at 12°C in AOAC medium not supplemented (WT and $\Delta casK/R$) or supplemented with PC (WT and $\Delta casK/R$). Each value is a mean of triplicate measurements with standard deviation calculated across triplicate measurements. FA nomenclature: iX = iso-FA with X number of carbons; aX = anteiso-FA with X number of carbons; nX = saturated FA with X number of carbons. When the FA was unsaturated, the symbol “:” prefixes the number of unsaturations into the FA chain.

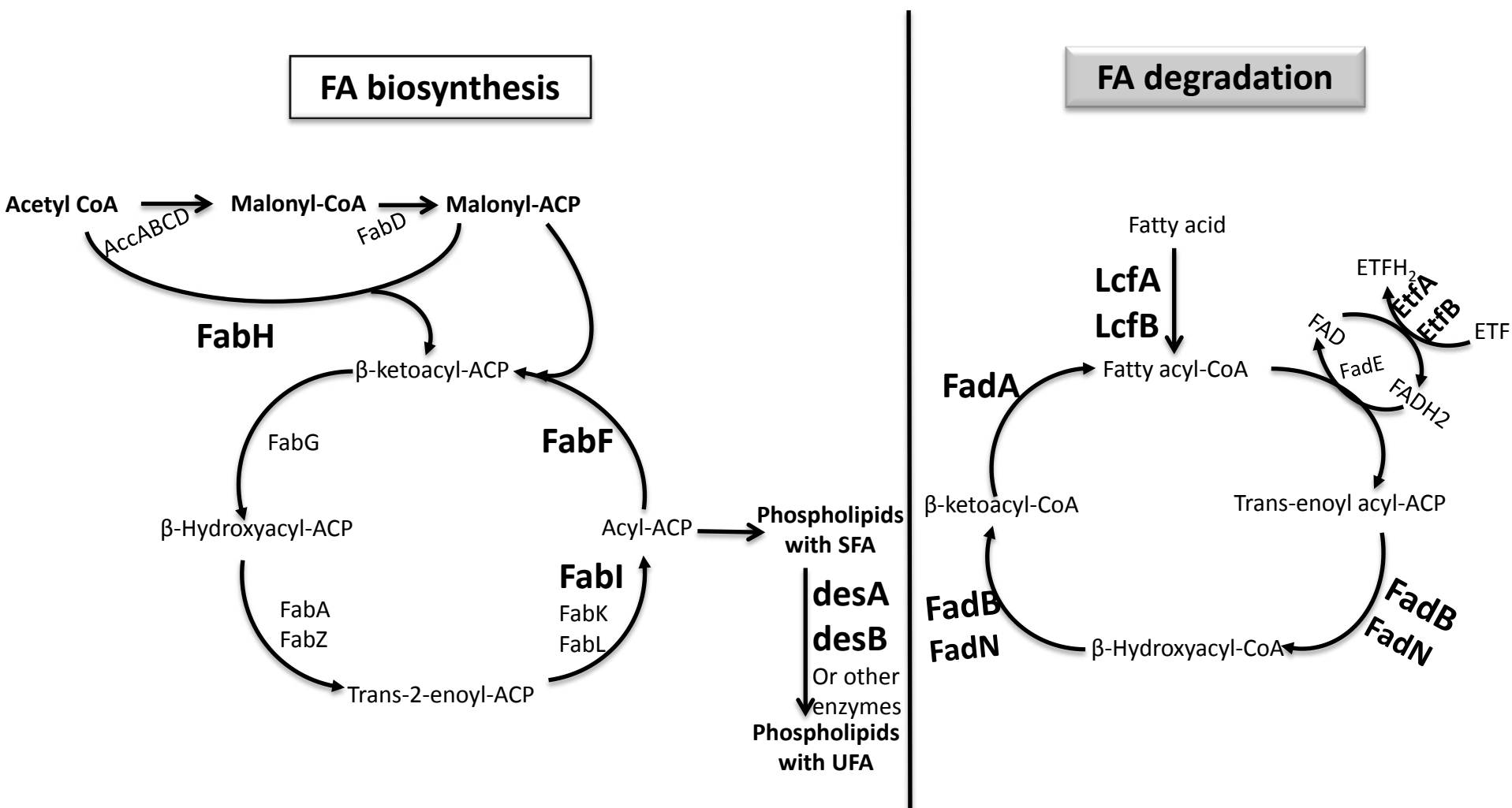


Figure S2: Genes down-regulated in the $\Delta casK/R$ vs WT strain at 12°C in FA biosynthesis and FA degradation. The genes down-regulated are represented in bold and bigger characters.

Résumé

Bacillus cereus sensu stricto (ss) est un pathogène alimentaire majeur représentant la 2^e cause de toxico-infection alimentaire en France en 2012. Cette espèce fait partie du groupe *Bacillus cereus sensu lato* (sl) constitué d'espèces ubiquitaires génétiquement très proches et incluant d'autres pathogènes comme *B. anthracis*, *B. thuringiensis* et *B. cytotoxicus*. Les souches de *B. cereus sl* sont d'autre part réparties en sept groupes phylogénétiques présentant des gammes de température de croissance variées et caractérisés par trois thermotypes principaux: thermotolérants, mésophiles, psychrotolérants. L'adaptation au froid des souches *B. cereus ss* est un mécanisme clé car il conditionne sa capacité à se développer dans les aliments réfrigéré pour atteindre des doses qui peuvent être dangereuse pour les consommateurs. Le but de cette étude a été d'étudier les mécanismes moléculaires impliqués dans l'adaptation au froid de la diversité de souches représentant *B. cereus sl*.

Nous avons mis en évidence que les gènes codant pour le système à deux composants CasK/R sont surexprimés à basse température. CasK/R s'est révélé être un système générique d'adaptation de *B. cereus sl* au froid, car son rôle a été mis en évidence lors de l'étude de quatre souches de thermotypes différents et leurs mutants isogéniques $\Delta casK/R$ respectifs. Une étude transcriptomique réalisée sur une souche ATCC 14579 et son mutant $\Delta casK/R$ a révélé que seize des gènes différentiellement exprimés en début de phase exponentielle et en phase stationnaire, à basse température, codent pour des protéines impliquées dans le métabolisme des acides gras. Nous avons mis en évidence le rôle de CasK/R dans la modification de la composition en acides gras membranaires via une augmentation de la proportion en acides gras insaturés lors de la croissance de *B. cereus* au froid. Par ailleurs, le gène codant pour la désaturase DesA, principalement responsable des insaturations des acides gras à basse température est régulée positivement par CasK/R au froid.

Nous avons également démontré que les gènes *casK/R* sont organisés en opéron avec un gène codant pour un régulateur RpiR-like. De manière originale, cet opéron est négativement régulé par CasK/R à basse température en phase stationnaire. Le promoteur individuel du *rpiR* est réprimé à basse température mais aussi à température optimale de croissance, ce qui suggère un rôle de CasK/R, même à température optimale.

Abstract

Bacillus cereus sensu stricto (ss) is a major foodborne pathogen representing the second cause of food poisoning in France in 2012. This species belongs to *Bacillus cereus sensu lato* (sl) consisting of ubiquitous species genetically close-related and including other pathogens such as *B. anthracis*, *B. thuringiensis* and *B. cytotoxicus*. The strains of *B. cereus sl* are divided into seven phylogenetic groups with various growth temperature ranges and characterized by three main thermotypes: thermotolerant, mesophilic, psychrotolerant. The *B. cereus ss* cold adaptation is a key mechanism because it determines *B. cereus* ability to grow in refrigerated foods and achieve doses that can be dangerous to consumers. The aim of this study was to study the molecular mechanisms involved in the cold adaptation of strains representing *B. cereus sl* diversity.

We demonstrated that the genes encoding the two component system CasK/R are overexpressed at low temperature. CasK/R was found to be a generic mechanism for *B. cereus sl* cold adaptation as its role was highlighted in the study of four strains with different thermotypes and their respective isogenic mutants $\Delta casK/R$. A transcriptomic study on a *B. cereus* ATCC 14579 strain and its $\Delta casK/R$ mutant strain revealed that sixteen of the genes differentially expressed in both early log phase and stationary phase at low temperature encode proteins involved in the fatty acids metabolism. We showed the role of CasK/R in the modification of the membrane fatty acid composition via an increase of the proportion of unsaturated fatty acids during growth of *B. cereus* at low temperature. Furthermore, the gene encoding the desaturase DesA, mainly responsible of the fatty acids unsaturation at a low temperature is upregulated by CasK/R at low temperature.

We also demonstrated that *casK/R* genes were organized in operon with a gene encoding a RpiR-like regulator. Interestingly, this operon is negatively regulated by CasK/R at low temperature in the stationary phase. The individual *rpiR* promoter is repressed by CasK/R at low temperature but also optimal growth temperature, suggesting also a role for CasK/R at optimal temperature.

Mots clés:

B. cereus, système à deux composants, froid, adaptation, acides gras