FcrX, a new global regulator of cell cycle in free living conditions and during symbiosis in Sinorhizobium meliloti

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ÉCOLE DOCTORALE
Sciences du végétal : du gène à l'écosystème (SEVE)

Titre : FcrX, un nouveau regulateur du cycle cellulaire en vie libre et pendant la symbiose chez *Sinorhizobium meliloti*

Mots clés : Cycle cellulaire, régulation génétique, différenciation cellulaire, symbiose fixatrice d'azote.

Résumé : *Sinorhizobium meliloti* est une bactérie capable d'établir une relation symbiotique avec les légumineuses des genres *Medicago*, *Melilotus* et *Trigonella*. Au sein des nodosités, *S. meliloti* fixe l'azote atmosphérique sous une forme assimilable par la plante en échange de sources carbonées. Au cours du processus symbiotique, *S. meliloti* se différencie en bactéroïdes. Ces bactéroïdes sont la forme cellulaire fixatrice de l'azote atmosphérique et se caractérisent par un élargissement cellulaire, une polyploïdie et une perméabilisation membranaire. La régulation du cycle cellulaire est étroitement liée au processus symbiotique. En effet, au niveau des bactéroïdes, le régulateur maitre du cycle cellulaire CtrA, responsable de l'inhibition de la réplication de l'ADN et l'activation de la division cellulaire (réalisée par la protéine FtsZ) sont absents. Cependant, le mécanisme par lequel sont régulés CtrA et FtsZ chez *S. meliloti* est encore mal connu à ce jour. Cette étude s'est focalisée sur un nouveau régulateur appelé FcrX «FtsZ cell cycle regulator X», essentiel chez *S. meliloti*. D'un côté, nous avons étudié le rôle de FcrX dans la régulation du cycle cellulaire en vie libre et son lien avec CtrA et FtsZ. Ainsi, en utilisant différentes approches nous avons pu mettre en évidence l'implication directe de FcrX dans la régulation négative de CtrA et FtsZ potentiellement via le dégradosome. D'un autre côté, nous avons investigué le rôle de FcrX pendant le processus symbiotique, et nous sommes arrivés à la conclusion que l'expression de FcrX est également essentielle dans un contexte de symbiose. De plus, une surexpression de ce régulateur est à l'origine d'un gain de masse chez la légumineuse *Medicago sativa*.

Title : FcrX, a new global regulator of cell cycle in free living conditions and during symbiosis in *Sinorhizobium meliloti*.

Keywords : cell cycle, genetic regulation, cell differentiation, nitrogen fixing symbiosis.

Sinorhizobium meliloti is bacterium that establishes a symbiosis with legumes plants such as *Medicago*, *Melilotus* and *Trigonella*. During symbiosis, *S. meliloti* fixes the atmospheric nitrogen into ammonia and in return the plant shares carbon sources with bacteria. During the symbiosis process, *S. meliloti* undergoes a drastic cellular change leading to an intracellular terminal differentiation into bacteroids, which are characterized by genome endoreduplication, cell enlargement and high membrane permeability. The bacterial cell cycle regulation is closely implicated in this process of differentiation. Indeed, in bacteroids, the bacterial regulator CtrA, which activates cell division (controlled by the constriction ring forming FtsZ) and inhibits DNA replication, and FtsZ are absent. However, the mechanism by which CtrA and FtsZ are downregulated is still unknown.

This study focuses on a new essential factor FcrX «FtsZ cell cycle regulator X» in *S. meliloti*. On the one hand, we studied the role of FcrX in cell cycle regulation in the free-living condition and its link with CtrA and FtsZ. Using several techniques we showed that FcrX is able to interact with FtsZ and CtrA and to downregulate their accumulation potentially via the degradosome. on the other hand, we investigated the role of FcrX during the symbiotic process, concluding that FcrX expression is also essential for the symbiosis establishment. Moreover, an overexpression of this regulator leads to a gain in dry mass of the symbiont partner *Medicago sativa*.

For my Father

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Résumé en français

1- Introduction :

a- Cycle cellulaire chez les alphaprotéobactéries *Caulobacter crescentus* et *Sinorhizobium meliloti*

Les études traitant du cycle cellulaire chez les bactéries sont d'une importance majeure depuis des décennies. En effet, la compréhension de ce mécanisme biologique nous permet de répondre à de vaste questions, notamment sur les mécanismes que déploie la bactérie afin de répliquer son ADN en tout intégrité et sur sa ségrégation d'une manière équitablement entre les cellules filles. En plus de l'intérêt de ces études sur le plan fondamentale, la compréhension des mécanismes qui régissent le cycle cellulaire sont utiles dans un contexte médical. En effet, le cycle cellulaire représente une cible intéressante pour le développement de molécules thérapeutiques et ceci afin de limiter la prolifération des bactéries pathogène.

L'étude du cycle cellulaire a connu un essor considérable grâce aux recherches effectuées sur le modèle bactérien *Caulobacter crescentus* ; une bactérie Gram-négative appartenant à la classe des alpha-protéobactéries. Le cycle cellulaire de cette bactérie donne naissance à deux cellules filles différentes; une grande cellule immobile grâce à un pédoncule à son pôle qui lui permet de se fixer aux surfaces et réplicative ainsi qu'une petite cellule mobile et non réplicative, qui dois se différencier en grande cellule afin d'entamer un nouveau cycle cellulaire. *C. crescentus* possède une caractéristique essentielle qui est de ne répliquer son ADN qu'une seule fois par cycle cellulaire, ceci permet donc de synchroniser les cellules et ainsi de pouvoir analyser chaque phase du cycle cellulaire individuellement (Barrows and Goley, 2023).

La régulation du cycle cellulaire chez *C. crescentus* est sous le contrôle de plusieurs régulateurs maitre (CtrA, GcrA, CcrM…). CtrA est un régulateur de réponse responsable de l'inhibition de la réplication de l'ADN et l'activation de la division cellulaire. CtrA, se fixe sur des séquences consensus au niveau de l'origine de réplication empêchant ainsi la fixation de la protéine DnaA responsable de l'initiation de la réplication de l'ADN. De plus, CtrA active la transcription des gènes *ftsQA* activant ainsi la division cellulaire. Ainsi, pour permettre une progression normale du cycle cellulaire, les niveaux d'expression de CtrA doivent être rigoureusement régulés au cours du cycle cellulaire. En effet, les niveaux d'expression de CtrA atteignent un maximum en phase G1 et pendant la transition S-G2. Cette régulation se manifeste au niveau transcriptionnel et post-traductionnel via la phosphorylation et la protéolyse (Collier, 2012).

CtrA n'est active que sous sa forme phosphorylée, cette phosphorylation est orchestrée par un phosphorelais pouvant être divisé en deux modules, le premier est composée de DivJ-PleC-DivK. Chez la cellule prédivisionnelle, l'histidine kinase DivJ est localisée au pôle de la future cellule immobile (*stalked*) et phosphoryle le régulateur de réponse DivK, tandis que la kinase bi-fonctionnelle PleC est située au pôle de la future cellule mobile (*swarmer*) et agit comme phosphatase de DivK, créant ainsi un gradient de DivK-P dans la cellule. Ainsi après la division cellulaire, la forme phosphorylée de DivK s'accumule dans la cellule immobile tandis que la cellule mobile ne contient que la forme déphosphorylée. Le deuxième module de phosphorylation centrale est composée du complexe DivL-CckA-ChpT-CtrA. Lorsque DivK n'est pas phosphorylé (dans le pôle du *swarmer*), l'histidine kinase bifonctionnelle CckA se lie à l'histidine kinase atypique DivL responsable de l'activité kinase de CckA et de sa localisation au niveau du pole *swarmer*. Une fois phosphorylé, CckA transfère son groupement phosphate à l'histidine phosphotransphérase ChpT, qui à son tour phosphoryle CtrA. Cependant, au pôle de la cellule immobile (*stalked*), DivK est phosphorylé et se lie à DivL. Ainsi, l'indisponibilité de DivL, associée à la fixation de c-di-GMP, synthétisé par la cyclase PleD, entraîne l'activation de la fonction phosphatase de CckA et de ChpT, par conséquent, la déphosphorylation de CtrA (Tsokos and Laub, 2012). Comme précisé en amont CtrA est également soumis à une régulation à travers la protéolyse, cette protéolyse est réalisée par un complexe de dégradation constitué de plusieurs acteurs : la protéase ClpP et son ATP-ase ClpX ainsi que des adaptateurs RcdA, CpdR et PopA qui permettent la reconnaissance et localisation de CtrA à la protéase (Mahmoud and Chien, 2018).

Le cycle cellulaire chez les alphaprotéobactéries semble être très similaire à celui observé chez les eucaryotes, notamment le régulateur maître CtrA et la distinction entre les différentes phases du cycle. En effet, il est intéressant de remarquer la similitude entre CtrA et les inhibiteurs des kinases cycline-dépendantes chez les eucaryotes, qui a l'instar de CtrA, interviennent de manière oscillatoire en déclenchant l'entrée dans la phase G1, agissant ainsi comme des points de contrôle pour les différentes phases du cycle cellulaire. L'étude du cycle cellulaire bactérien est donc essentielle pour la compréhension de ce processus biologique en général et pour la compréhension de la régulation du cycle cellulaire eucaryote (Amon, 1998).

Sinorhizobium meliloti est une bactérie Gram-négative appartenant également à la classe des *Alphaprotéobacteria*. Cette bactérie possède deux modes de vie, libre dans le sol et en symbiose avec les plantes légumineuses. En vie libre, le cycle cellulaire de *S. meliloti* est similaire à celui décrit chez son homologue *C. crescentus*. En effet, on observe une seule

réplication de l'ADN par cycle cellulaire ainsi qu'une asymétrie des cellules filles au niveau morphologique et physiologique avec une grande cellule réplicative et une petite cellule qui doit se différencier en grande cellule pour entamer un nouveau cycle cellulaire. Le régulateur maître CtrA occupe également une position clé dans la régulation du cycle cellulaire et de ce fait est régulé comme chez *C. crescentus* au niveau transcriptionnel et post-traductionnel (De Nisco *et al.*, 2014; Pini *et al.*, 2015).

Contrairement au modèle *C. crescentus*, très peu d'informations concernant la régulation du cycle cellulaire sont disponibles. Cependant, des études ont démontré la conservation de la même logique générale mais aussi la conservation de la plupart des régulateurs connus chez *C. crescentus*. En effet, tous les facteurs cités ci-dessus sont conservés chez *S. meliloti*. Cependant, quelques différences doivent être notées. *S. meliloti*, contrairement à *C. crescentus*, possède une seconde histidine kinase CbrA et deux copies du régulateur de réponse CpdR (CpdR1 et CpdR2), CpdR1 étant la copie régulée par le cycle cellulaire. On note également la présence de trois copies de la protéase ClpP (ClpP1, ClpP2 et ClpP3). En outre, *S. meliloti* ne code pas pour la protéine adaptatrice PopA et, contrairement à *C. crescentus*, le c-di-GMP semble avoir perdu son rôle clé dans la régulation du cycle cellulaire. De plus, chez *S. meliloti*, CtrA inhibe la réplication de l'ADN de manière indirecte, contrairement à ce qui est observé chez *C. crescentus.* Cependant le mécanisme impliqué n'est pas encore identifié à ce jour. Une différence supplémentaire est également à noter chez *S. meliloti*, en effet, CtrA active la division cellulaire d'une manière indirecte en inhibant la transcription du système *minCDE,* qui a pour fonction d'inhiber la polymérisation de la tubuline-like FtsZ autre part qu'au niveau du site de division (Corbin *et al.*, 2002). FtsZ étant la protéine qui forme l'anneau de constriction et qui permet la division cellulaire. Chez *S. meliloti* deux copies de FtsZ sont présentes (FtsZ1 et 2), cependant la fonction de FtsZ2 est à ce jour inconnue.

b- Symbiose fixatrice d'azote chez le modèle *Sinorhizobium meliloti- Medicago sativa*

L'atmosphère est composée de 80% d'azote atmosphérique. Malgré son abondance, il constitue un facteur limitant pour la croissance des plantes. En effet, les plantes ne possédant pas d'enzymes capables de fixer l'azote atmosphérique, cette dernière dépendent des ressources azotées présentent dans le sol (Rascio and Rocca, 2008). Ainsi, pour pallier cette indisponibilité de l'azote et afin de faire face à la demande alimentaire croissante, conséquence d'une augmentation démographique importante, les fertilisants chimiques sont massivement utilisés. Cependant, ces derniers sont à l'origine d'une pollution environnementale importante. De ce

fait, trouver une alternative aux fertilisants est plus que nécessaire (Erisman *et al.*, 2013). Une des solutions proposée est de recourir à la fixation biologique de l'azote et ceci à travers l'utilisation de bactéries capables d'interagir avec les plantes légumineuses afin d'établir une symbiose fixatrice d'azote.

S. meliloti est capable d'interagir avec les plantes légumineuses des genres *Medicago, Melilotus* et *Trigonella.* Cette interaction donne naissance à un organe symbiotique appelé nodosité. Au sein de la nodosité, *S. meliloti* fixe l'azote atmosphérique en ammoniaque, qui est ensuite converti en ammonium par la plante, une forme assimilable par les plantes et en échange la plante hôte procure une niche et des sources carbonées à la bactérie. L'établissement de cette relation symbiotique se base sur un échange de signaux chimiques entre la plante hôte et la bactérie symbiotique. Dans le modèle *Medicago-S. meliloti,* l'absence d'azote dans le sol induit chez la plante hôte une sécrétion de molécules signales connues sous le nom de flavonoïdes. Ces molécules sont détectées par *S. meliloti,* en se liant à son régulateur de réponse NodD1. Ce dernier, active l'expression de gènes codant des enzymes responsables dans la biosynthèse de facteurs Nod, des lipo-chito-oligosaccharides. Les facteurs Nod interagissent à leur tour avec la plante hôte afin d'enclencher la biogenèse de la nodosité et la formation du fil d'infection. La bactérie est attirée par chimiotaxisme vers la plante (Mergaert *et al.*, 1997; Peck *et al.*, 2006). Ce premier contact induit une courbure du poil racinaire qui piège une microcolonie bactérienne. Cette dernière finit par être internalisée et les rhizobia progressent à travers le cordon d'infection puis sont libérés dans la nodosité à l'intérieur des symbiosomes par un phénomène d'endocytose (Skorupska *et al.*, 2006).

Durant ce processus symbiotique, la bactérie *S. meliloti* subit un programme de différenciation terminale menant à la formation d'un bactéroïde, la forme cellulaire fixatrice d'azote. Le bactéroïde est une cellule caractérisée par un important élargissement cellulaire, une endoreduplication et une perméabilisation membranaire. Des études ont démontré que le processus de différentiation est induit par l'effet des peptides NCRs (Mergaert *et al.*, 2006) . Les NCRs sont des peptides antimicrobiens produit par la plante hôte au niveau de la nodosité. Chez *M. truncatula,* près de 700 peptides différents ont été identifiés. Le peptide NCR247, l'un des peptides les mieux étudiés, interagit avec des cibles intracellulaires bactériennes, notamment des protéines ribosomiques, la tubuline-like FtsZ, GroEL et RpoH (Farkas *et al.*, 2018). De plus, le traitement d'une culture de *S. meliloti* avec le NCR247 a montré une baisse d'expression du régulon de CtrA et de FtsZ ainsi que la production d'un phénotype similaire aux bactéroïdes observés au niveau de la nodosité (Penterman *et al.*, 2014). En effet, l'absence

de CtrA ainsi que FtsZ est parfaitement cohérente avec le phénotype du bactéroïde, qui consiste en une levée de l'inhibition de la réplication de l'ADN et l'absence de division cellulaire (Pini *et al.*, 2015). Cependant, le mécanisme par lequel les NCRs induisent la différentiation en bactéroïde est inconnu. Ainsi, le cycle cellulaire de la bactérie symbiotique semble étroitement lié au processus de différentiation et par conséquent à une symbiose efficace. On pourrait citer l'effet de la délétion de CpdR qui induit une surexpression de CtrA et ainsi une symbiose déficiente (Kobayashi *et al.*, 2009).

2- Résultats

a- Découverte d'un nouveau régulateur du cycle cellulaire chez *Sinorhizobium meliloti*

Dans le cadre de cette thèse, nous avons investigué le rôle d'un nouveau régulateur du cycle cellulaire chez *S. meliloti* appelé « FcrX », *fcrX* est un gène de 351 paires de base codant une petite protéine de 116 acides aminés. Ce dernière a suscité notre intérêt de par ses caractéristiques intéressantes. En effet, un criblage génétique réalisé sur le mutant Δ*divJ* précédemment introduit a révélé que FcrX est un régulateur potentiel de CtrA, ce qui lui confère un lien direct avec le cycle cellulaire. De plus, la séquence codante de *fcrX*, partage la même séquence intergénique avec le gène codant CtrA et tous deux sont orientés dans des sens opposés, ce qui peut également suggérer un lien fonctionnel entre ces deux protéines. La délétion de FcrX conduit à une létalité de la bactérie, ainsi les études entreprises afin de comprendre la fonction de ce facteur ont été en partie réalisées à l'aide d'une souche de déplétion de *fcrX*.

La souche de déplétion a été construite en délétant la copie chromosomique de *fcrX* et en exprimant une copie ectopique portée par un plasmide. La copie de *fcrX* est sous le contrôle d'un promoteur p*lac* inductible par l'ajout d'IPTG. L'observation sous microscope du phénotype de la souche de déplétion de *fcrX*, a révélé un défaut de croissance qui se manifeste par la production de petites cellules « *minicells* » dépourvues d'ADN. Ceci suggère un arrêt de réplication de l'ADN ainsi qu'un excès de division cellulaire. De ce fait, FcrX pourrait être impliqué dans la régulation du cycle cellulaire. Pour vérifier cette hypothèse un Western blot a été réalisé afin d'évaluer les concentrations des protéines CtrA et FtsZ1et 2 en absence de FcrX. Nous avons choisi de mesurer ces protéines en particulier car ces dernières sont les protagonistes dans les processus de la régulation du cycle cellulaire ainsi que la division cellulaire, respectivement. Le Western blot a révélé l'effet négatif de FcrX sur l'accumulation

de FtsZ1 et 2 ainsi que CtrA. En effet, en absence de FcrX, une accumlation des protéines CtrA et FtsZ a été observée. Ceci démontre que FcrX est un régulateur négatif de ces protéines.

Une prédiction de la structure tridimensionnelle de FcrX réalisée avec l'outil AlphaFold a révélé une structure riche en hélice alpha dépourvue de domaine se liant à l'ADN ou d'un motif enzymatique. La prédiction de la structure protéique de FcrX laisse penser à un mode d'action par interaction physique avec les cibles. Cette hypothèse a été vérifiée à travers des tests d'interaction (double hybride bactérien et une colonne d'affinité). Ainsi, la colonne d'affinité a révélé une interaction entre FcrX et les protéines CtrA, FtsZ1 et 2. Ces résultats ont été confirmés à travers le double hybride bactérien en utilisant le modèle *Escherichia coli* qui a également montré un résultat positif avec ces trois protéines.

Ensuite, à l'aide de construction de mutants de complémentation nous avons été en mesure d'identifier la séquence promotrice de *fcrX*. Après l'analyse de cette séquence promotrice de 289 paires de base, nous avons pu identifier la présence d'une boîte CtrA; une séquence d'ADN consensus au niveau de laquelle CtrA se fixe, et ceci afin d'activer ou d'inhiber la transcription du gène en aval. Afin de tester la potentielle implication de CtrA dans la régulation transcriptionnelle de *fcrX*, nous avons réalisé une mutation au niveau de cette séquence, et analysé l'effet sur la transcription de *fcrX*. En utilisant un gène rapporteur nous avons constaté qu'en présence de cette mutation empêchant la fixation de CtrA, le gène rapporteur n'est plus exprimé contrairement à la séquence contrôle utilisée dont la box de CtrA est intègre. Ces résultats ont été appuyés par la mesure du niveau de transcription du gène *fcrX* par qRT-PCR et qui en absence de CtrA a également montré une diminution de transcription. Ainsi, ces observations indiquent l'existence d'une boucle de rétrocontrôle entre CtrA et FcrX. Ce type de régulation est à la base des phénomènes biologiques cycliques afin de permettre l'expression oscillatoire de certains régulateurs.

-Localisation subcellulaire de FcrX et ses cibles chez *S. meliloti*

Afin d'avoir plus d'informations sur la fonction de la protéine FcrX chez *S. meliloti*, nous avons investigué la localisation subcellulaire de cette dernière. Pour ce faire, nous avons construit des souches de déplétion de *fcrX*, exprimant une fusion traductionnelle C-terminale de FcrX avec une protéine fluorescente YFP (Yellow Fluorescent Protein). Les observations au microscope ont révélé une localisation au septum et une localisation moins fréquente au niveau des pôles. Pour vérifier si la localisation de FcrX était cohérente avec celle de ses cibles, nous avons également construit des fusions traductionnelles C-terminale de CtrA et les protéine FtsZ

avec une protéine fluorescente CFP (Cyan Fluorescent Protein). Dans le cas de la protéine CtrA, une fusion au niveau C-terminal prévient sa dégradation et stabilise la protéine menant à son accumulation. L'accumulation de CtrA dans la cellule est toxique. Ainsi, les 15 derniers acides aminés de CtrA sont ajoutés à la suite de la séquence codant la protéine CFP. Cette séquence correspond à une étiquette reconnue par la protéase afin de permettre sa dégradation. Ensuite cette construction a été exprimée dans une souche dont la copie chromosomique de CtrA a été délétée et ceci afin de vérifier la fonctionnalité de la nouvelle copie. Les observations sous microscope ont montré, comme attendu, une localisation au septum pour FtsZ 1 et 2 tandis que CtrA a montré une localisation au pôle cellulaire. Ces observations sont en accord avec la localisation de FcrX, ce qui renforce nos résultats d'interactions précédemment énoncés.

- **Analyse de la conservation de FcrX chez les bactéries**

Afin d'analyser la conservation de FcrX chez les bactéries nous avons réalisé une recherche d'orthologues en utilisant l'outil *Bidirectional Best Hit* (BBH). Ce dernier a révélé la présence d'orthologues chez plusieurs rhizobia (*Bradyrhizobium japonicum, Rhizobium leguminosarum*), le pathogène humain *Brucella abortus* et le phytopathogène *Agrobacterium vitis*. Ces orthologues ont été testés pour leur capacité à complémenter la délétion de *fcrX* chez *S. meliloti.* Nous avons également testé un homologue éloigné de *fcrX* chez *C. crescentus*, qui code pour la protéine flagellaire FliJ, dont la position génomique est similaire que celle du gène *fcrX*. En effet, il est intéressant de noter que la synténie *fcrX-ctrA* est largement conservée parmi ces bactéries, ce qui suggère une possible conservation de la fonction de *fcrX*. Les tests de complémentation ont révélé que l'expression des orthologues provenant de *R. leguminosarum* et de *B. abortus* était capable de complémenter la fonction de FcrX chez *S. meliloti*, ce qui implique une conservation fonctionnelle de FcrX. Cependant, les orthologues provenant de *B. japonicum, A. vitis* et l'homologue FliJ de *C. crescentus* n'ont pas été en mesure de complémenter la délétion de *fcrX* chez *S. meliloti.* Ainsi, cette analyse montre que d'un point de vue fonctionnel, chaque copie de *fcrX* a acquis des fonctions spécifiques indépendamment de la distance phylogénétique par rapport à *S. meliloti,* comme par exemple chez *A. vitis* (une espèce proche de *S. meliloti*) qui n'est pas capable de complémenter la fonction de FcrX chez *S. meliloti* contrairement à la copie provenant de *B. abortus*.

- **Etude du rôle de FcrX pendant la symbiose avec *M. sativa***

Ensuite, nous avons analysé le rôle de FcrX durant la symbiose avec la légumineuse *M. sativa.* Pour ce faire, nous avons inoculé des plantes de *M. sativa* avec une souche de déplétion

de *fcrX*. Afin d'obtenir des conditions avec différents niveaux d'expression de FcrX, nous avons constitué plusieurs groupes de plantes que nous avons arrosé avec des concentrations croissantes de l'inducteur IPTG. L'analyse a été effectué 5 semaines après inoculation, une mesure du poids sec des plantes. De plus, la capacité des bactéries à coloniser la nodosité a été étudiée par microscopie confocale. Enfin, le contenu en ADN et la taille des bactéroïdes déterminés par cytométrie en flux ont montré que les plantes inoculées avec les souches exprimant le moins de FcrX présentaient des défaut symbiotiques. Ces observations ont été comparés avec la référence inoculée avec une souche sauvage. Cependant, plus les concentrations d'IPTG étaient élevée plus le phénotype observé se rapprochait de celui de la référence. Ainsi, l'expression de FcrX est primordiale pour l'établissement de la symbiose. Cet effet peut être lié à une implication directe de FcrX dans le processus symbiotique ou bien être lié à l'état physiologique de la bactérie qui est désavantagée (défaut général de fitness) et ainsi ne permet pas une colonisation efficace de la nodosité.

Nous avons également testé la surexpression de FcrX pendant la symbiose. Les plantes de *M. sativa* inoculées avec une souche surexprimant FcrX ont montré un gain de croissance et un poids sec supérieur en comparaison avec les plantes inoculées avec une souche sauvage. Les observations microscopiques de la nodosité afin d'évaluer la capacité à coloniser celle-ci et les analyses de la taille et contenu en ADN des bactéroïdes par cytométrie en flux, n'ont pas révélé de différences significatives entre la condition de surexpression et la souche sauvage. De ce fait, l'effet de la surexpression de FcrX pourrait être expliqué par la dégradation plus rapide de CtrA et des protéines FtsZ et par conséquent une différenciation prématurée en bactéroïdes. Ainsi, la surexpression de FcrX apporte un avantage de croissance à travers l'amélioration de la symbiose entre les deux partenaires. La manipulation de FcrX par voie biotechnologique pourrait donc constituer une alternative prometteuse aux fertilisants, afin de promouvoir une agriculture à bas intrants et/ou qui permettrait d'augmenter les rendements en agriculture.

b- Lien entre FcrX et le dégradosome chez *Sinorhizobium meliloti*

Les résultats précédents ont révélé l'implication de FcrX dans la régulation négative de CtrA et les protéines FtsZ1 et 2. Cependant, le mécanisme par lequel FcrX agit reste inconnu. Pour répondre à cette question, des tests d'interaction ont été réalisés afin d'explorer d'éventuelles interactions qui pourraient expliquer la fonction de FcrX. Une colonne d'affinité a révélé une interaction entre FcrX et la protéase ClpP1. Ce dernier résultat a été confirmé par double hybride bactérien, qui nous a permis par la même occasion de vérifier l'interaction de FcrX avec les adaptateurs RcdA et CpdR.

Une localisation subcellulaire des acteurs du dégradosome a été réalisée. Pour ce faire, nous avons exprimé chez *S. meliloti* une fusion traductionnelle C-terminale de ClpP1 et ClpX avec une protéine fluorescente CFP. Les observations au microscope ont montré une localisation au niveau du septum et au pôle cellulaire pour les deux protéines. Ces résultats sont cohérents avec la localisation de FcrX et les résultats des tests d'interaction. Ces observations suggèrent que FcrX pourrait avoir un lien fonctionnel avec le complexe du dégradosome et donc être impliqué dans le processus de protéolyse. L'interaction de FcrX avec ces facteurs pourrait aussi être le résultat de sa propre dégradation.

Nous avons ensuite vérifié la nature du lien entre FcrX et le complexe du dégradosome. Pour ce faire, un Run off a été réalisé afin de mesurer la stabilité de FcrX en comparaison avec CtrA et ceci dans une souche sauvage et une souche de déplétion de RcdA. Ces expériences ont démontré que la concentration de FcrX reste invariable pendant la période de l'expérience soit 75 minutes, en comparaison avec CtrA dont la concentration diminue après 15 minutes de mesure. Ces résultats démontrent que FcrX ne constitue pas une cible du dégradosome contrairement à CtrA mais pourrait avoir un rôle d'adaptateur, qui amènerait ces cibles CtrA, FtsZ1 et FtsZ2 à la dégradation.

Un Western blot nous a permis de détecter la présence de FcrX au niveau des bactéroïdes contrairement à CtrA et aux protéines FtsZ. Ainsi la détection de FcrX pourrait être liée à la stabilité inhérente à la protéine qui lui permet de persister au niveau des bactéroïdes. Alternativement, un facteur produit par la plante pourrait stabiliser la protéine pour favoriser la dégradation de CtrA et les protéines FtsZ. Comme cité précédemment, la différenciation cellulaire en bactéroïdes est assurée par des peptides sécrétés par l'hôte, appelés NCR, qui induisent la disparition des protéines CtrA et FtsZ et ceci via un mécanisme encore inconnu. L'interaction physique du peptide NCR247 avec de multiples protéines intracellulaires telles que FtsZ, GroEL, RpoH et les protéines ribosomales a déjà été rapportée (Farkas et al., 2014). Ainsi, il est raisonnable de proposer FcrX comme une cible potentielle des peptides NCR qui pourraient interagir physiquement avec FcrX et de stabiliser la protéine.

Ainsi, pour identifier un potentiel lien entre les peptides NCR et FcrX, nous avons réalisé dans un premier temps un test de sensibilité au peptide NCR247 des souches de *S. meliloti* exprimant FcrX à différents niveaux. Cette expérience nous a révélé un effet de la concentration de FcrX sur la sensibilité de *S. meliloti* au peptide NCR247. En effet, lorsque la protéine FcrX est surexprimée, la sensibilité de *S. meliloti* au peptide NCR247 augmente. Ce résultat confirme que la protéine FcrX est d'une certaine manière liée à l'effet du peptide

NCR247 (et peut-être d'autres peptides NCR non caractérisés à ce jour). La protéine FcrX peut être liée directement au peptide comme nous l'avons proposé ci-dessus, ou bien l'effet peut être indirect. En effet, la surexpression de FcrX induit la diminution des protéines CtrA et FtsZ, qui comptent parmi les cibles du peptide NCR247. Pour vérifier cette hypothèse, nous pourrions réaliser un test d'interaction tel qu'une colonne d'affinité.

3- Conclusion générale

La découverte du nouveau régulateur du cycle cellulaire "FcrX" chez *Sinorhizobium meliloti*, est une étape supplémentaire qui nous permet de mieux comprendre la régulation du cycle cellulaire chez ce modèle bactérien. De plus, au vu de la conservation de FcrX chez des espèces pathogènes et de son implication dans la régulation du cycle cellulaire et de la division cellulaire, ce régulateur pourrait devenir une cible potentielle pour la conception de molécules thérapeutiques.

L'effet positif de la surexpression de FcrX sur la croissance de la plante, a révélé une fois de plus le lien étroit entre le cycle cellulaire bactérien et le processus de symbiose. Ainsi, il est important de mieux comprendre la régulation du cycle cellulaire chez les bactéries rhizobia afin de pouvoir améliorer la symbiose. Le travail conduit pendant cette thèse a permis de mettre en lumière un nouveau facteur pouvant être exploité dans le cadre de l'amélioration de la symbiose entre *S. meliloti- M. sativa.* De plus, la conservation de la protéine FcrX chez certains rhizobia, permettrait de tester l'effet de ce facteur chez d'autre légumineuses modèles ou d'intérêt agronomique. Ainsi, à long terme des souches surexprimant ce facteur pourraient être utilisées afin de promouvoir la croissance des légumineuses. De ce fait, utiliser les légumineuses pour enrichir les sols en azote, et ainsi pallier aux dégâts engendrés par l'utilisation excessive des fertilisants.

Cette étude ouvre ainsi de nombreuses perspectives sur la recherche de nouveaux régulateurs, qui agiraient directement sur le cycle cellulaire bactérien afin d'améliorer la symbiose légumineuses-rhizobia. Néanmoins, des expériences supplémentaires devrons être conduites afin de mieux comprendre la mécanistique par laquelle FcrX agit sur ses cibles ainsi que la potentielle implication de la plante dans la stabilisation de ce régulateur.

ABBREVIATIONS

--A--

16S rRNA: 16Svedberg ribosomal Ribonucleic Acid
A: Adenosine
ADP: Adenosine DiPhosphate
Asp: Aspartate
ATP: Adenosine TriPhosphate

--B--

BacTH: Bacterial Two Hybrid
BBH: Bidirectional Blast Hit
bp: base pair

--C--

C: Cytosine
CA: Catalytic and ATP-binding
CAP: Catabolite Activator Protein
c-di-GMP: Cyclic di-Guanosine MonoPhosphate
CDK: Cyclin-Dependent Kinase
CFP: Cyan Fluorescent Protein
cm: Centimeter
CSC: Circular Sliding Clamps

--D--

DAPI: 4′,6-DiAmidino-2-PhenylIndole
DHp: Dimerisation and Histidine phosphotransfer
DIC: Differential Interference Contrast
DNA: DeoxyriboNucleic Acid
DNF1: Defective in Nitrogen Fixation
dpi: days post-inoculation
DUE: DNA Unwinding Element

--E—

EGFP: Enhanced Green Fluorescent Protein

--G--

G: Guanosine
G1 phase: Gap1 phase
G2 phase: Gap2 phase
GTPase: Guanosine TriPhosphate

--H--

HEPES: 4-(2-HydroxyEthyl)-1-Piperazine EthaneSulfonic acid
HGT: Horizontal Gene Transfer
His: Histidine
His6: Histidine 6
HK: Histidine Kinase
HO: homothallism

--I--

ICE : Integrative and Conjugative Elements
IPTG: IsoPropyl-ß-D-ThioGalactopyranoside
IRLC: Inverted-Repeat-Lacking Clade

--K--

kDa: kiloDalton

--L--

LB: Lysis Broth

--M--

MOPS: 3-(N-MOrpholino)PropaneSulfonic acid

--N--

nC: number of Cycle
NCR: Nodule-specific Cysteine-Rich
ncRNA: Non coding RNA
NF: Nodulation Factor

--O--

OCS: One Component System
oriC: origin Chromosome
OD_{600}: Optical Density 600

--P--

PC: Phase Contrast
PCR: Polymerase Chain Reaction
PI: Propidium Iodide
P*lac*: Promotor lactose
ppGpp:
ppm: Parts per minute
Rpm: Revolutions per minute

--R--

REC: Receiver domain
RNA: Ribonucleic Acid

RNAi: Ribonucleic Acid interference
RNAP: Ribonucleic Acid Polymerase
RR : Response Regulator
rRNA: ribosomal RNA

--S--

S phase: DNA Synthesis phase
SDS-PAGE: Sodium Dodecyl-Sulfate PolyAcrylamide Gel Electrophoresis
SEM: Scanning Electron Microscopy
sp.: species
sRNA: small RNA
SSB: Single-Strand DNA-Binding protein
ssDNA: Single stranded DNA
ssp.: subspecies
SSU: Small SubUnit

--T--

T: Tyrosine
TBD: Terminal Bacteroid Differentiation
TCS: Two Component System
TEM: Transmission Electron Microscopy
tetR: tetracyclin Resistance
Tn-Seq: Transponson Sequencing
Tris: Tris (Hydroxymethyl) Aminomethane)
TSS: Transcription Start Site

--U--

UTR: UnTranslated Region

--W--

WT: Wild-Type

--Y--

YEB: Yeast Extract Broth
YFP: Yellow Fluorescent Protein

Others

3D: 3 Dimension
µm: micrometer

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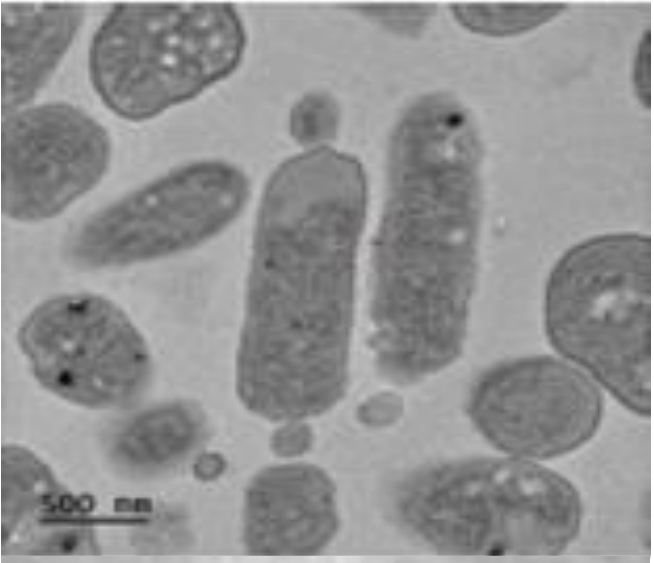
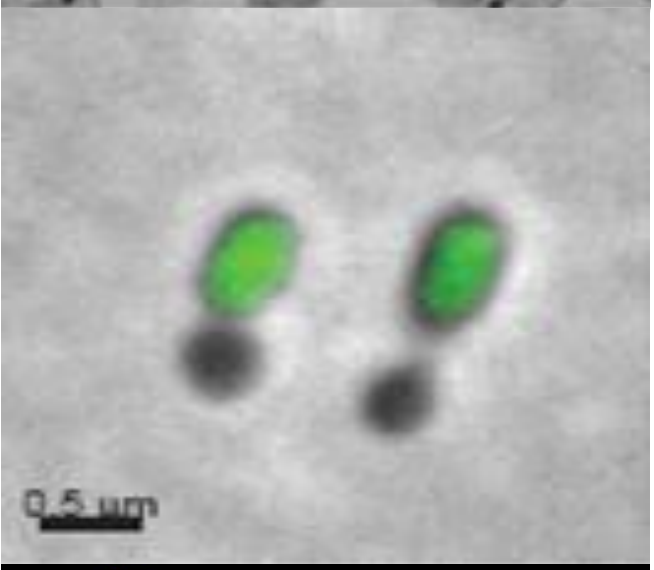
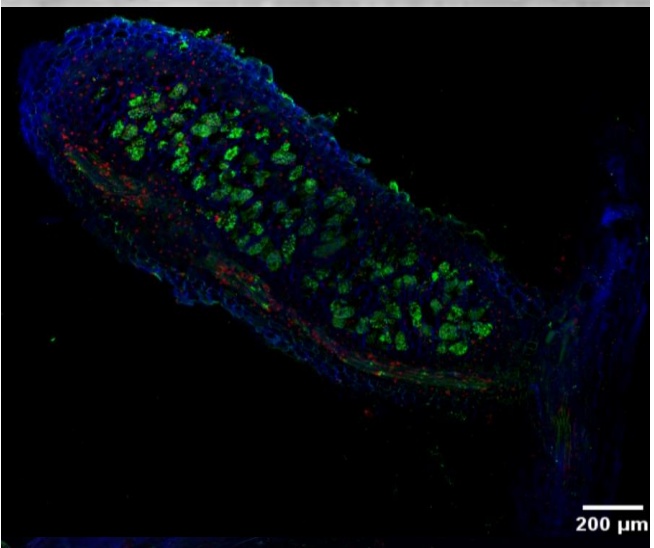






I- Introduction

1- The kingdom of bacteria

Humans have been always astonished by the huge diversity of living organisms cohabiting the planet. To this extent, many scientists and philosophers have dedicated their lives to decipher the mysteries of life, while ignoring for many centuries the existence of an invisible world, as vast as the visible one. This latter is represented by microorganisms including bacteria, archaea, protozoa, algae, and fungi. A microorganism by definition designates an organism usually invisible to the naked eye. However, recent discoveries have shown that there are some exceptions such as the giant bacteria *Epulopiscium fishelsoni* (600µm long), *Thiomargarita namibiensis* (750µm) and lastly *Thiomargarita magnifica* (1cm) (Schulz and Jørgensen, 2001; Volland *et al.*, 2022).

a- The discovery of the new domain: bacteria

The discovery of bacteria is assigned to the pioneer of microbiology, Antoni van Leeuwenhoek in 1674 Leeuwenhoek observed small organisms in the rainwater that he called back then “animalcules” (Figure 1). These, later on, were defined as protozoa and bacteria. This shattering discovery has revolutionized the way we see life. This hidden in plain sight life opened a brand-new field in science. From then until our days, microbiologists have still been trying to understand this form of life, even with the development of sophisticated tools and a better comprehension of their biology, however many questions remain unanswered.

Since the late 19th century, the microbiologists have based their observations on pure bacterial isolates using Agar-containing Petri dishes. However, due to the difficulty faced to reproduce adequate environmental conditions in the laboratory, many of them have remained uncultured. Nevertheless, with the development of molecular biology, culture-independent methods for bacterial identification such as 16S ribosomal RNA (rRNA) sequencing is nowadays available and widely used. These methods have helped to expand considerably our knowledge of the diversity of the bacterial kingdom (Austin, 2017).

The bacterial kingdom diversity is humongous and constitutes, the second most important biomass on earth after plants in particular embryophytes (Bar-On *et al.*, 2018). Thanks to their adaptive and versatile metabolism, bacteria thrive in almost every environment: soil, water, sediments, even the extreme ones such as deep-sea hydrothermal vents, arctic glaciers or acidic lakes. These extremophile bacteria are able to cope with the hostile conditions surrounding them, for example by modifying the envelop composition and expressing proteins

and enzymes that can tolerate extreme abiotic conditions (Reed *et al.*, 2013; Siliakus *et al.*, 2017) or even modifying the classical metabolic pathways (Bräsen *et al.*, 2014).

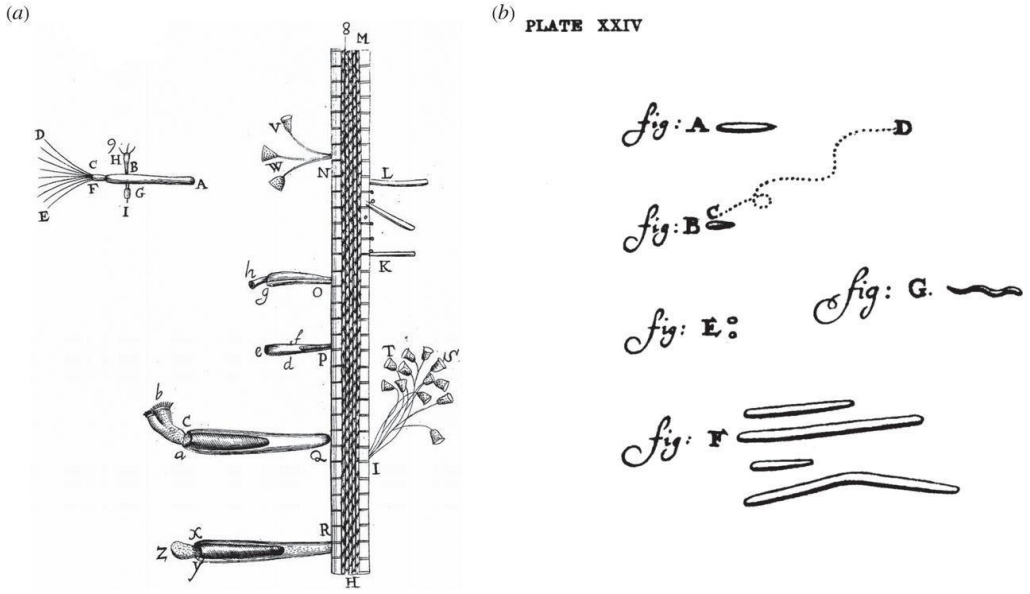


Figure 1: Schemes from Antoni van Leeuwenhoek letters dating from 1674.

(a) Rotifers, hydra and vorticellids associated with a duckweed root, from a Delft canal. (b) Figures of bacteria observed from Leeuwenhoek's mouth sample; the dotted line from C to D refers to the motility of the bacteria (Lane, 2015).

b- The bacterial classification through history

At the beginning of the species classification, only Plants (non-motile) and Animals (motile) kingdoms were recognized. Since the bacteria seemed to share more common characteristics with plants than animals, they were classified in the plant kingdom. In 1899, Ernst Haeckel and collaborator shed the light on the differences between organisms containing a nucleus and those that are devoid of it. Known afterward as eukaryotes and prokaryotes respectively, thus resulting in the creation of a third kingdom "Protista" that grouped all organisms lacking the nucleus structure including bacteria (Davis *et al*, 1967). In 1977, Carl Woese and Wolfe used the 16S rRNA gene sequence to revise the classification, which resulted in reclassifying Archaea in a distinct domain leading to three systems domain (Eukarya, Bacteria and Archaea) (Rappé and Giovannoni, 2003). Moreover, the discovery of Archaea led the scientists to reconsider the bifurcation of the tree of life previously recognized by all. Today, the three domains (Eukarya, Bacteria and Archaea) and six kingdoms (Plantae, Protista,

Animalia, Fungi, Archaea, Eubacteria) classification is well admitted and accurate considering all the knowledge available and accumulated during these decades of research (Figure 2).

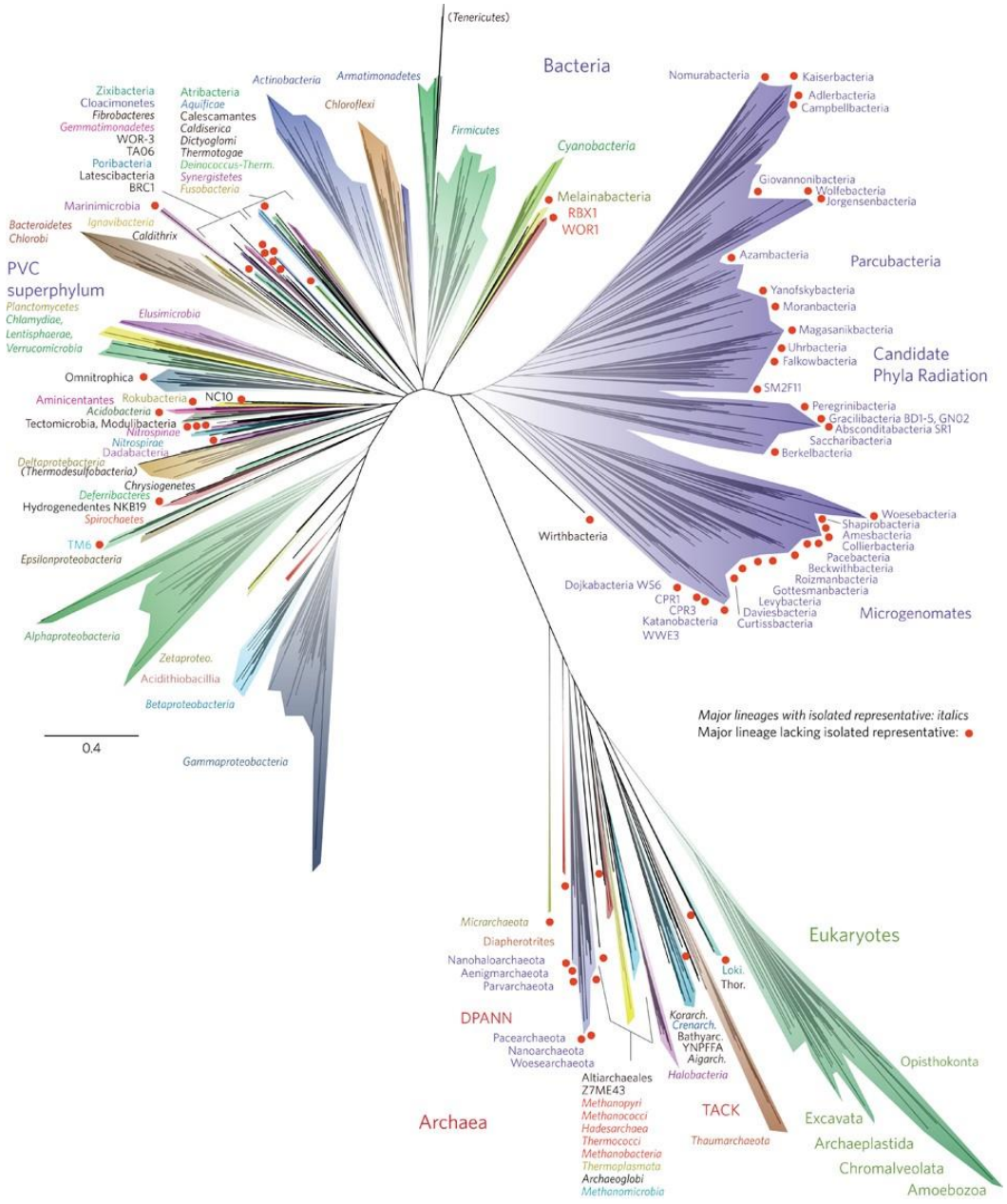


Figure 2: Phylogenetic tree of life.

A phylogenetic tree including 92 bacterial phyla, 26 archaeal phyla and 5 Eukaryotic super groups. The well-characterized lineages are written in italics; non-characterized lineages are designed with non-italics names and red dots. The tree was generated by aligning small subunit (SSU) rRNA genes available from the genomes of the organisms included in the ribosomal protein data set (Hug *et al*., 2016).

The bacteria are microscopic unicellular organisms differently shaped. This feature can also be used for their classification. The most common cell shapes are spherical (cocci) like *Staphylococcus aureus*, rod-shaped (bacilli) like *Escherichia coli*, helical (spirilli) like *Spirillum volutans* (Figure 3A-C). The German naturalist and botanist Ferdinand Cohn introduced the method organizing bacteria into genera and species based on their size and shape. However, to properly classify bacteria, this approach was insufficiently informative but still essential for the study of bacterial physiology until these days. In 1884, the bacteriologists Carl Friedlander and Hans Christian Gram, invented a staining procedure that allowed them to divide bacteria into two major groups according to their envelope composition (Popescu and Doyle, 1996). Thus, we can distinguish Gram-negative bacteria, whose envelope is composed of a thin peptidoglycan cell wall surrounded by an outer membrane, and Gram-positive bacteria, which are composed of a thicker peptidoglycan cell wall without an outer membrane (Sizar and Unakal, 2022) (Figure 3D).

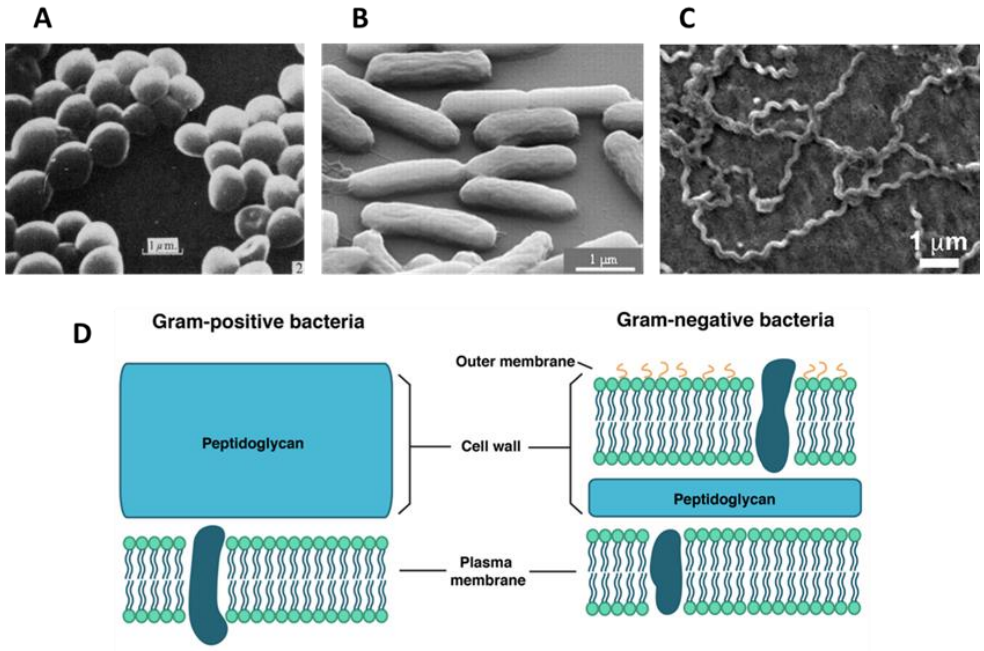


Figure 3 : Scheme representing different bacterial shapes and envelop composition.

A) image representing a Scanning Electronic Microscopy (SEM) picture of *Staphylococcus aureus*, showing the round shape and the organization in grape-like clusters, which is characteristic of this bacterium (Greenwood and O'grady, 1972). B) SEM image of *Escherichia coli* on silicon platelets showing a bacillus shape (Hartmann *et al.*, 2010). C) SEM image of *Leptospira biflexa* in liquid medium showing a spirillum (helicoidal corkscrew-like shape) (Golding *et al.*, 2016) D) Schematic representation of a cross-section of the cell wall and cell membrane of Gram-positive (left) and Gram-negative (right) bacteria (Rahman *et al.*, 2021).

2- Deciphering the environmental changes by bacteria

Even if bacteria are microscopic organisms and structurally simple, compared to the eukaryotes, their physiology can be very complex. Responses and adaptations rely often on sophisticated re-adaptation of gene expression programs. Therefore, gene regulation in bacteria is surprisingly intricated. More in details, in order to adapt to environmental changes, like deficiencies in nutrients, brutal temperature changes or predators, bacteria have to consequently reprogram their gene expression. To do so, the environmental changes have to be sensed by the bacteria and the signal transduced inside the cell, in order to respond adequately. The mechanism involved in this sensing in known as signaling transduction, this process is mainly supported by one- (OCS) or two-component system (TCS).

a- The signal transduction systems

The reversible phosphorylation of proteins is an important biological process in eukaryotes as well as in bacteria but long overlooked. It was finally brought to the light by the Nobel prizes Edwin Krebs and Edmond Fischer in 1954. Whereas the first protein kinase cascade was discovered in 1968. The protein phosphorylation is a posttranslational modification that consists in adding a phosphate group on a specific amino acid (ex. tyrosine, histidine). This reaction is conducted by an enzyme called kinase or phosphotransferase while the removal of the phosphate group is realized by a phosphatase enzyme. These posttranslational modifications impact the targeted proteins activity by inducing conformational changes for example (Cohen, 2002; Kyriakis, 2014; Zhang *et al.*, 2021)

The OCS are the simplest transduction systems, composed by a unique protein containing both an input domain that senses the stimuli (sensor) and an output domain responsible for the response (review) and are mostly intracellular, for instance the transcription regulator CAP and LacI in *E. coli* (Ulrich *et al.*, 2005). Oppositely, TCS are fine-tuned systems composed of two separated proteins; an input domain, which is a histidine kinase protein (HK) and an output domain called response regulator protein (RR) (Mitrophanov and Groisman, 2008) (Figure 4). The number of OCS in bacteria outcompetes the TCS, more interestingly the inputs diversity is greater in the former than the latter. It is admitted that the appearance of OCS precedes that of TCS, which evolved from OCS. Indeed, the TCS seems to give an obvious advantage hence it links the extracellular sensing to the intracellular sensing by a phosphotransfer relay. The TCS are widespread among organisms, as they are found in bacteria, archaea and in some eukaryotes such as plants, fungi and protozoa. However, bacteria are the organisms that use them the most

and it is considered that this system appeared first in bacteria and was spread by horizontal transfer to other biological kingdoms (Hirakawa *et al.*, 2020).

The functioning of signal transduction systems is based on the process introduced in the previous paragraph, starting from the autophosphorylation of the HK (using ATP as phosphate-donor) upon a specific signal, followed by a phosphate transfer (acting as a posttranslational signal) between the HK and the RR. The modular composition of the TCS testify of the signal transduction complexity. Indeed, to ensure a fast and adequate response the TCS have to be specific and precise (Ulrich *et al.*, 2005). For example, the EnvZ-OmpR TCS controls the expression of the porin genes *ompF* and *ompC* in *E. coli* (Qin *et al.*, 2001). The bacteria exhibit a more complex version of TCS that counts additional phosphotransfer proteins, called phosphorelay. For instance, the phosphorelay that regulates the sporulation in the bacterium *Bacillus subtilis* was the first identified by Burbulys *et al.* in 1991. This latter is composed by a four-step His-Asp-His-Asp phosphorelay. First of all, the kinases KinA, KinB, or KinC are phosphorylated, then the phosphoryl group is transferred to the response regulator Spo0F, then to the phosphotranspherase Spo0B and finally to the response regulator Spo0A (Appleby *et al.*, 1996). The phosphorelay may sense the input signals with greater level of control and accuracy through supplementary nodes and checkpoints. In addition, the phosphorelay complexity may avoid detrimental crosstalk between distinct signaling systems but rather provides junction points for benefic cross regulation (Appleby *et al.*, 1996; Hoch and Varughese, 2001; Stephenson and Hoch, 2002).

The histidine kinase protein is composed of a variable sensor domain at the N terminus, which spans the cell membrane allowing it to sense environmental or internal stimuli and a conserved C terminus that comprises two domains; a dimerization histidine-phosphotransfer (DHp) domain and the catalytic and ATP-binding (CA) domain (Zschiedrich *et al.*, 2016). The response regulator comprises an invariable N-terminal receiver (REC) domain containing a conserved aspartic acid residue and a variable output domain at the C terminus, which is usually a binding site on the deoxyribonucleic acid (DNA, see next section), that activates or represses transcription initiation of target genes (Gao et *al*, 2019).

TCS are implicated in the regulation of numerous biological processes, such as response to stresses, cell cycle regulation and motility. Their main function is to regulate gene expression at the transcription level, as most of gene regulation happens at this stage. In most cases, gene expression is regulated at the transcription initiation stage (promoter recognition and

transcription initiation). However, expression modulation can intervene at all levels; at the post-transcriptional level, where non-coding RNAs and RNA-binding proteins are involved in the stabilization or the interruption of RNA translation initiation (Van Assche *et al.*, 2015). Once the translation has started the processes of elongation and termination are also subject to regulation depending on regulatory signals. Finally, the newly synthetized protein can also be post-translationally regulated by adding supplemental modifications, such as phosphate (through a phosphorylation usually done by the signal transduction systems as seen in the previous paragraphs) to modulate their activity, or by adding a sugar (through glycosylation) to promote the adhesion of bacteria to the host during colonization (Macek *et al.*, 2019). The newly synthetized proteins can also be cleared from the cell by proteolytic degradation.

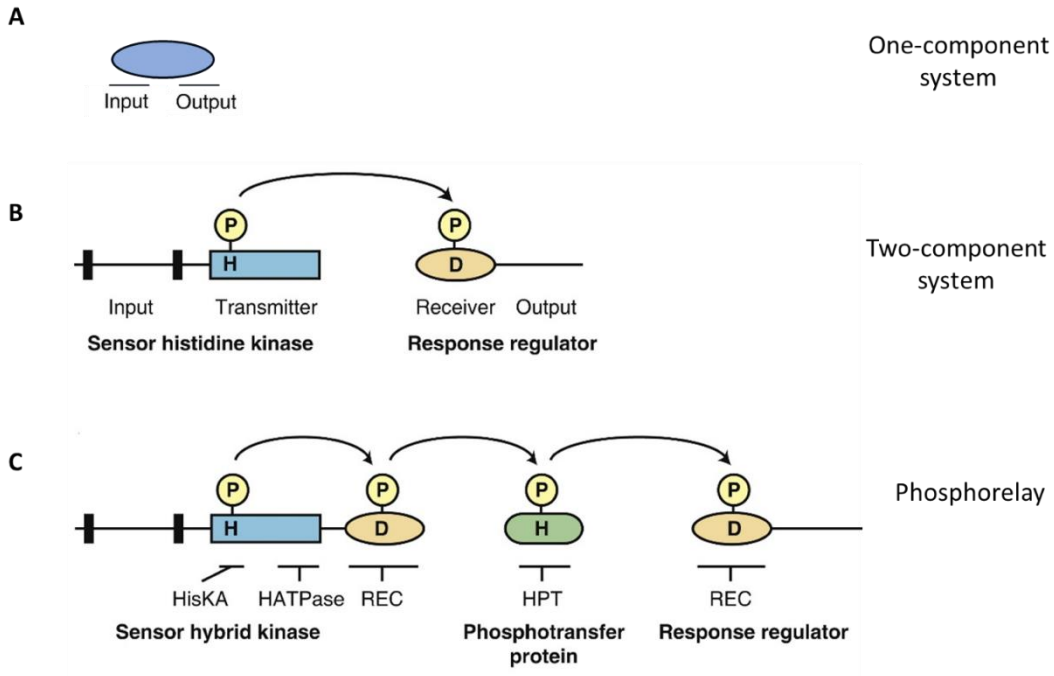


Figure 4: Schematics of the proteins and domains implicated in signal transduction in prokaryotes.

Histidine kinase domains are indicated by rectangles, receiver domains by ovals, histidine-containing phosphotransfer domains by rounded rectangles, and transmembrane domains by black bars. Sites of phosphorylation upon histidine (H) and aspartic acid (D) residues are indicated. (A) One-component system is the simplest transduction system composed by a single protein that senses and respond to the stimuli in the same manner than the two-component system. (B) Simple two-component system that employs two distinct proteins; a histidine kinase and a response regulator. (C) Multi-step phosphorelay that employs a hybrid histidine kinase with both histidine kinase and receiver domains, a histidine-containing

phosphotransfer protein, and a response regulator. Regions covered by consensus domains (HisKA, HATPase, HPT, and REC) are indicated. (Figure adapted from Schaller *et al.*, 2011).

b- The discovery of deoxyribonucleic acid

The DNA is the molecule that encodes the genetic information of all living organisms. To decode this information the DNA goes through a transcriptional process that results in RNA production. The RNA is then translated into proteins by ribosomes by a three-letters (codon) alphabet (Matsushita and Kubitschek, 1975).

The DNA was isolated for the first time in 1869 by the Swiss doctor Friedrich Miescher. Miescher initial plan was to isolate proteins from Human leucocyte cells, but he accidently precipitated a molecule that was not sensitive to proteases and called it nuclein. *"In my experiments with weakly alkaline solutions, when neutralizing the solution, I could obtain precipitates that could not be dissolved either in water, acetic acid, very dilute hydrochloric acid, or in solutions of sodium chloride, and which thus could not belong to any of the hitherto known proteins."* (Miescher 1869). Since then, scientists were enthralled by this discovery and many questions raised, notably about the possible function of the DNA and its composition.

In 1950, Erwin Chargaff discovered that the same molecule of DNA contains equal amounts of adenine (A) and thymine (T) and equal amounts of cytosine (C) and guanine (G), and that proportion of (A-T) and (C-G) varies between different species, supporting that DNA may be a carrier of genetic information. This hypothesis was confirmed by the work conducted by Alfred Hershey and Martha Chase in 1952. One year later, Francis Crick and James Watson characterized the double helix structure of DNA based on X-ray analyses made by Rosalind Franklin and Maurice Wilkins. However, cracking the genetic code of life was possible only in 1960 (Dahm, 2008).

c- Gene transcription in bacteria

Regulation of transcription is linked to the knowledge of the mechanism of transcriptional activation. The RNA polymerase is the enzyme responsible of the transcription of genes; it is composed by several units (ββ'α2ω) forming the core enzyme. To initiate gene transcription in bacteria, the core enzyme of RNA polymerase requires another protein called the sigma factor to constitute a complete holoenzyme (Figure 5). The sigma factor was first purified 54 years ago by Burgess and Travers. In this study, they already suggested the involvement of sigma

factors in the recognition of transcription starting sites (promoter sequence) and this by binding the RNA polymerase (RNAP) to the DNA (Burgess et al., 1969).

Based on their structure, the sigma (σ) factors can be classified into two different families; the sigma 54 (σ54) and the sigma 70 (σ70) family. The σ54 when present is for example implicated in the nitrogen assimilation. The σ70 family, the largest one, is divided into four groups; the group 1 that comprises the housekeeping sigma factor, which is expressed in normal conditions, groups 2 and 3 that are involved in the general response to stresses or virulence and finally the group 4 involved in the extracellular stress responses (Figure 5). Their expression depends on the environmental conditions thereby each sigma factor recognize a specific promoter sequence. This specificity and diversity of factors and the cognate sequences they recognize contributes to the establishment of a fine-tuned regulation system that guaranties a plasticity of adaptation to the bacteria.

To start DNA transcription, the RNAP binds to specific sequences upstream the coding sequence, overall known as promoter. The promoter is comprised of two conserved consensus sequences the -35 and the -10 (TATAAT, known as TATA box), as their name reflects their position (in bp) on the genome with respect to the gene transcription start site (TSS) and are bound by specific protein domains of the RNAP. Strong promoters contain additional sequences (UP elements) that help the RNAP to bind more efficiently to the promoter sequence. While the weak promoters, share less similarity with the consensus sequence of the strong promoters and lack additional above-cited sequences.

In addition to the RNA polymerase consensus sites and UP elements, other consensus sequences can be found in the promoter sequence. DNA-binding proteins, known as transcription factors, recognize several specific consensus sites. These latter can interfere positively or negatively with transcription initiation. Depending of their binding sequence position in the promoter, the transcriptional regulators can activate or inhibit the transcription of the downstream genes, some of them exhibiting both functions depending on the gene. The transcription factors (and their regulation) are the link between the gene expression and the environmental conditions, hence their expression and activity therefore many of them have to be regulated in return (Stekel and Jenkins, 2008; Gottesman, 2019). To do this, several regulation points exist, such as, for example, i. the affinity of the transcriptional factors for the DNA sequence they bind, ii. the regulation of their expression itself, that can occur when their target genes need to be expressed or iii. posttranslational level by phosphorylation or

proteolysis. Indeed, some transcription factors (such as, the response regulators of TCSs) are phosphorylated or dephosphorylated by their cognate TCS kinases modulating their function.

The expression of some genes is energetically expensive. In order to avoid wasting energy, bacteria regulate gene expression and invest only in those that are needed. As explained before, bacteria face a plethora of environmental changes hence the adaptation to these latter has to be fast and efficient (Stekel and Jenkins, 2008). The ideal way is to act on the early steps of gene expression. The transcription being the first step leading to gene expression, many regulation mechanisms are known to occur at this level, especially at the transcription initiation even before the RNA is produced.

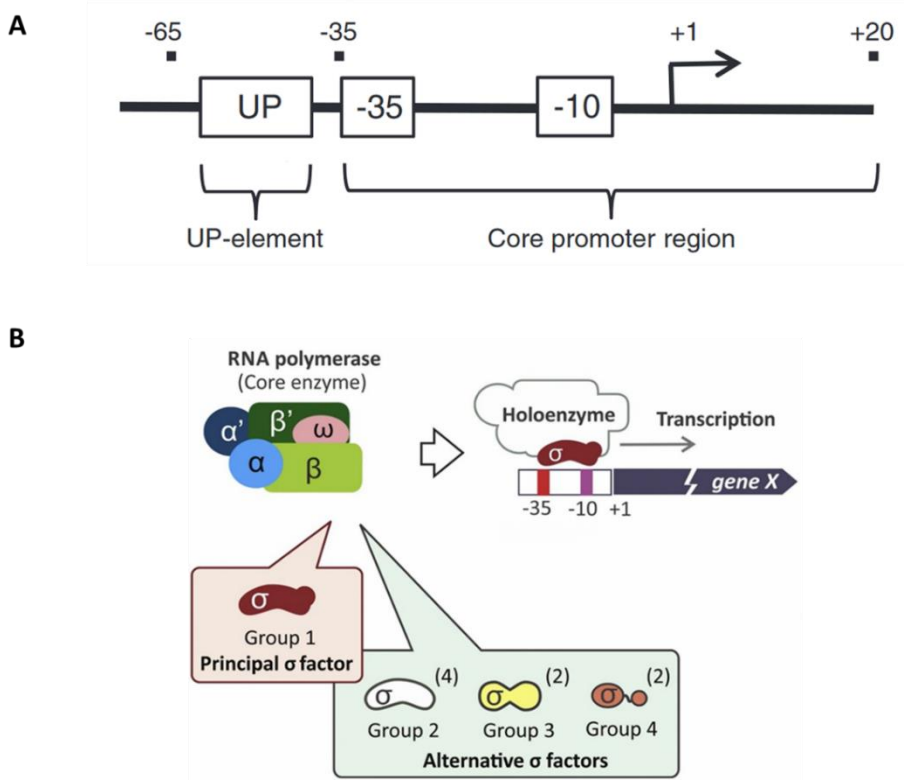


Figure 5: The bacterial promoter structure and the RNA polymerase.

(A) Schematic representing the promoter sequence and its different regions (UP element and the core promoter region that comprises the -35 and -10 boxes) (adapted from Rhodius *et al.*, 2011). (B) Schematic representing the bacterial RNA polymerase (RNAP) holoenzyme. The bacterial RNAP core enzyme is composed of subunits (α2ββ'ω) together with the dissociable σ factor forms the holoenzyme. The dissociable σ factor recognize specific promoter elements and helps the binding of the RNAP during the initiation of transcription. Several groups of σ factors exist, the group 1 represents the housekeeping σ factor while the groups 2, 3 and 4 are alternative ones expressed in particular stress conditions (Paget, 2015). (Figure adapted from Srivastava *et al.*, 2020).

3- Regulation of transcription drives the bacterial cell cycle

Gene transcription is essential for the homeostasis of all organisms and is the first step to many biological processes. It was demonstrated that a normal progression of the cell cycle is tightly linked to the oscillatory expression of transcriptional factors, these latter will switch Off and On a specific process and act as check points in both eukaryote and bacteria cell cycle (Bertoli *et al.*, 2013; Bristow *et al.*, 2014). All living organisms have to reproduce to ensure their persistence in the environment. The reproduction in bacteria is achieved by binary fission. This biological process was first studied in the bacterial model *Escherichia coli* (Donachie, 1993). However, with the development of new genetics tools and the cultivability of numerous bacteria, this knowledge has been extended to more models, for example *Caulobacter crescentus* a Gram-negative bacterium (Govers and Jacobs-Wagner, 2020),or mycobacteria (Trojanowski *et al.*, 2015; Bandekar *et al.*, 2020). The cell cycle in bacteria can be divided in three continuous events: a first event (C period or S-phase) during which the DNA is replicated into two accurate genomic copies. Second event (D period or G2-phase), between the end of replication and segregation of DNA represented by the cell division, giving birth to two distinguishable daughter cells. Finally, the third event (B period or G1-phase) corresponds to the cell growth phase taking place between the end of cell division and the initiation of DNA replication (Wang and Levin, 2009).

a- DNA replication

The accurate replication of the DNA supported by control systems such as DNA repair is a perquisite to ensure the inheritance of the genetic information that defines the organism through generations. However, some variability can occur, as a consequence of point mutations, horizontal gene transfer and recombination. These modifications are the powerhouse of species evolution. Moreover, the genetic variability is at the basis of the Darwinian Theory consisting in the natural selection of the more adapted species to the environmental changes, which was demonstrated by the fluctuate test conducted by Salvador Luria and Max Delbruck in 1943 (Luria and Delbrück, 1943; Steinert *et al.*, 2000; Murray, 2016).

The DNA replication is investigated since decades, mostly in the bacterium model *E. coli*. This process involves a number of proteins that assemble in a fine-tuned machine known as the replisome, which is conserved in all three domains of life but with notable differences. The origin (oriC) is the starting point of replication where two forks of replication form and proceed in a bidirectional manner around the chromosome, which is usually circular in bacteria.

The well-accepted DNA replication model is semi-discontinuous process where, due to functioning of DNA polymerase, the leading strand (5'-3') is synthetized in a continuous manner while the lagging strand (3'-5') is synthetized discontinuously in ~2 kb Okazaki fragments. First, the initiator protein DnaA, an AAA+ protein binds to conserved sequences (DnaA boxes) located in the origin near an AT-rich DNA unwinding element (DUE). The binding of DnaA proteins creates a torsion on DNA inducing the melting of the DUE sequence and promotes the recruitment of the replisome complex consisting of helicase, primase, SSB (single-strand binding protein), CSC (circular sliding clamps), a pentameric clamp loader and DNA polymerase. This protein complex operates in hierarchical manner. When the origin is in an open state, the primosome composed by the helicase and the primase intervene, the helicase separates the double stranded DNA and the SSB proteins immediately binds to the fragile single stranded DNA. Thus, to prevent the formation of secondary structure that may interrupt the replication machinery. The primase synthetizes the primers (a short RNA sequence) which are essential for the activity of the DNA polymerase. Then, the circular sliding clamps are loaded to the single strand DNA by the clamp loader that binds the primed ssDNA, the CSC in turn will load the DNA polymerase that proceeds in the DNA synthesis. As the two forks meet at the termination site, the newly synthetized chromosomes are segregated leaving room for cell division (Figure 6) (O'Donnell *et al.*, 2013; Oakley, 2019).

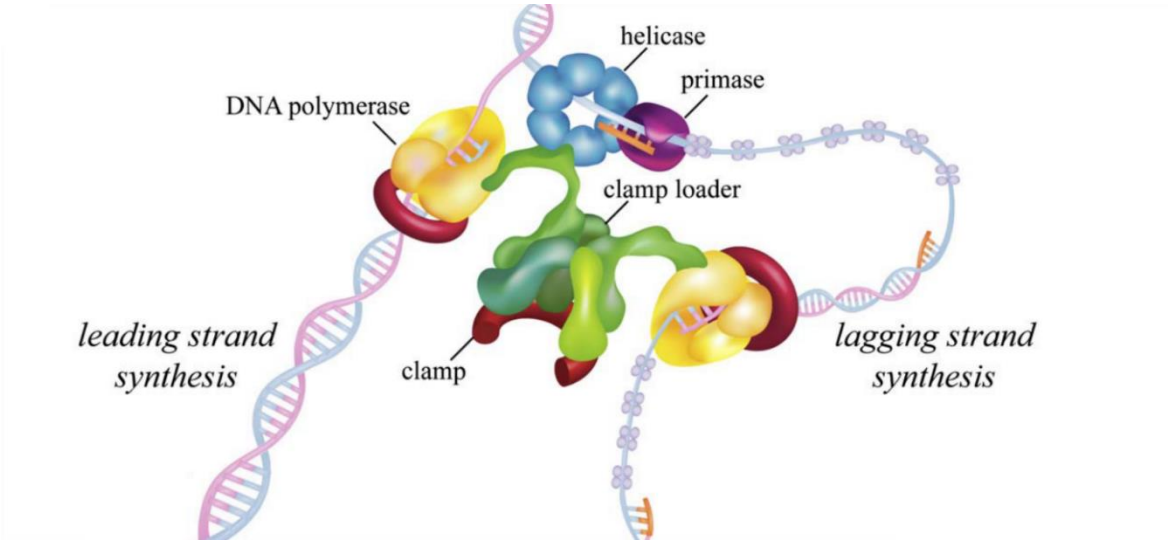


Figure 6: Scheme representing a DNA fragment being replicated by the replisome.

The replisome is composed by a primase, a helicase, a clamp and clamp loader, a single-strand binding protein and a DNA polymerase. On the right, the lagging stand synthesis and the Okazaki fragments are represented. On the left, the continuous synthesis of the leading stand is represented (Langston and O'Donnell, 2006).

b- Cell division

The cell division is the process in which a (mother) cell divides and gives birth to two (daughter) cells following the complete replication of DNA. The cell division is ensured by the divisome, a complex of multiple proteins. In *E. coli* the core divisome is composed by FtsZ, FtsA, ZipA, FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI, and FtsN. The key protein of the division process FtsZ is a highly conserved tubulin-like protein. FtsZ is a composed of four domains: a variable N-terminal, a variable spacer domain, a conserved core region containing a GTPase domain that allows its polymerization to form a Z ring at the septum, and a C terminus that binds to and recruits other proteins from the divisome machinery homeostasis, responsible for the activation of the cell wall synthesis resulting in the septum constriction. Thus, despite the key role of FtsZ, the cell division requires an important number of additional, often essential, proteins that, in opposition to FtsZ, are less conserved among bacteria. In *E. coli*, FtsZ localizes at the division site prior to the subsequent proteins of the complex, and it is anchored to the membrane, thanks to FtsA and ZipA. FtsZ, ZipA and FtsA form a dynamic ring structure known as protoring, which in turn recruits other proteins, FtsN, FtsQLB and FtsWI. These latter proteins are involved in the peptidoglycan layer synthesis, which allows the constriction of the

cell envelope, and ultimately the cell constriction (Figure 7) (Adams and Errington, 2009; Blaauwen *et al.*, 2017; Söderström and Daley, 2017; Mahone and Goley, 2020).

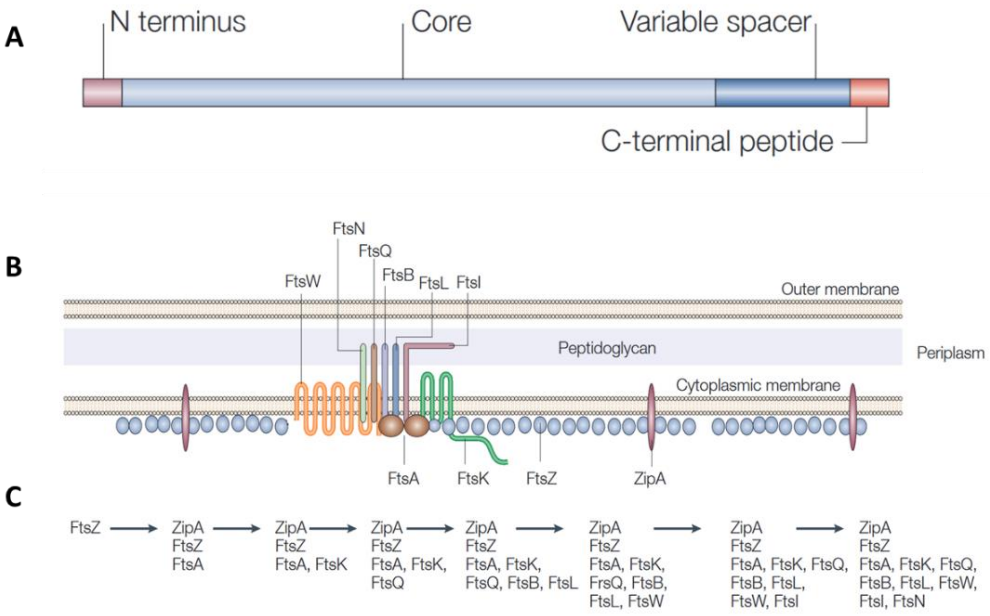


Figure 7: Scheme of the Z ring protein FtsZ and the divisome complex.

(A) Structure of the FtsZ tubulin-like protein (B) Scheme representing the core proteins composing the divisome machinery in the model *Escherichia coli.* These proteins are recruited to the division site in hierarchical manner as shown in the panel (C) the order of protein intervention is essential for a proper cell division (Margolin, 2005).

The bacteria mark the division site differently depending on the species. This site can be located more or less at the middle of the cell, resulting in symmetrical or asymmetrical division of daughter cells. For example, *E. coli* and many other Gram-negative bacteria position their septum precisely at the mid-cell (50 +/-3%) while in *Caulobacter crescentus* the septum is a bit off the middle of the cell (53 +/-0.1%). We can also observe a sloppy septum position in the Gram-positive bacteria *Corynebacterium glutamicum* and *Mycobacterium tuberculosis* (25-75%) (den Blaauwen, 2013). The positioning of the Z ring in the bacteria is clearly subject to a fine regulation. The main protein of the cell division FtsZ is spatially regulated in *E. coli* by the MinCDE system, which is highly conserved among bacteria. It consists of three proteins MinC, MinD and MinE. MinD and MinC together prevent the polymerization of FtsZ while MinE promotes the FtsZ polymerization at the mid-cell by dislodging MinCD. These proteins oscillate from a cell pole to another, resulting in a high concentration at the cell pole preventing

the septation. Thus, the septum formation can occur only at the mid-cell (Corbin *et al.*, 2002; Ramm *et al.*, 2019). In addition to the MinCDE system, the position of the Z ring is consolidated by the presence of nucleoid occlusion system mediated by SlmA. This latter binds to specific site of the genome to ensure the formation of the septum only after the perfect segregation of the two copies of the genome. In *C. crescentus,* the MinCDE system is absent. However, a cell pole localized protein named MipZ is responsible of the inhibition of FtsZ polymerization (Thanbichler and Shapiro, 2006). Interestingly, while in *E. coli* FtsZ levels seem to be constant during the cell cycle, in *C. crescentus*, an additional regulation layer exists. Indeed, FtsZ levels change during the cell cycle progression, a result of transcriptional and posttranslational regulation (Fatima *et al.*, 2022).

The bacterial growth implies enlargement of the cell size, therefore this energy-expensive process requires a fine control. Indeed, when the environmental conditions and nutrient availability are favorable, the bacteria grow in size and divide. Whereas, when the conditions are hostile to the bacteria, growth is slowed or even arrested to save energy such as cellular quiescence in *Mycobacterium tubercolusis* or dormancy, through spore production, in *Bacillus subtilis* for instance (Rittershaus *et al.*, 2013). The doubling time can vary depending on the species and growth condition (nutrient availability). Bacteria such as *E. coli* and *B. subtilis* are considered as fast-growing bacteria, while *C. crescentus* and *Rhodobacter sphaeroides* are known as slow-growing bacteria. In both cases, the timing of the processes of DNA replication and cell division are constant. In rich media, the doubling time of fast-growing bacteria can be decreased by overlapping several rounds of DNA replication during cell growth (Kornberg and Baker 1992; Helmstetter 1996). In nutrient-limited conditions and in slow-growing alphaproteobacteria the opposite is noticed, where only one round of DNA replication occurs at once (Reyes-Lamothe and Sherratt, 2019) (Figure 8).

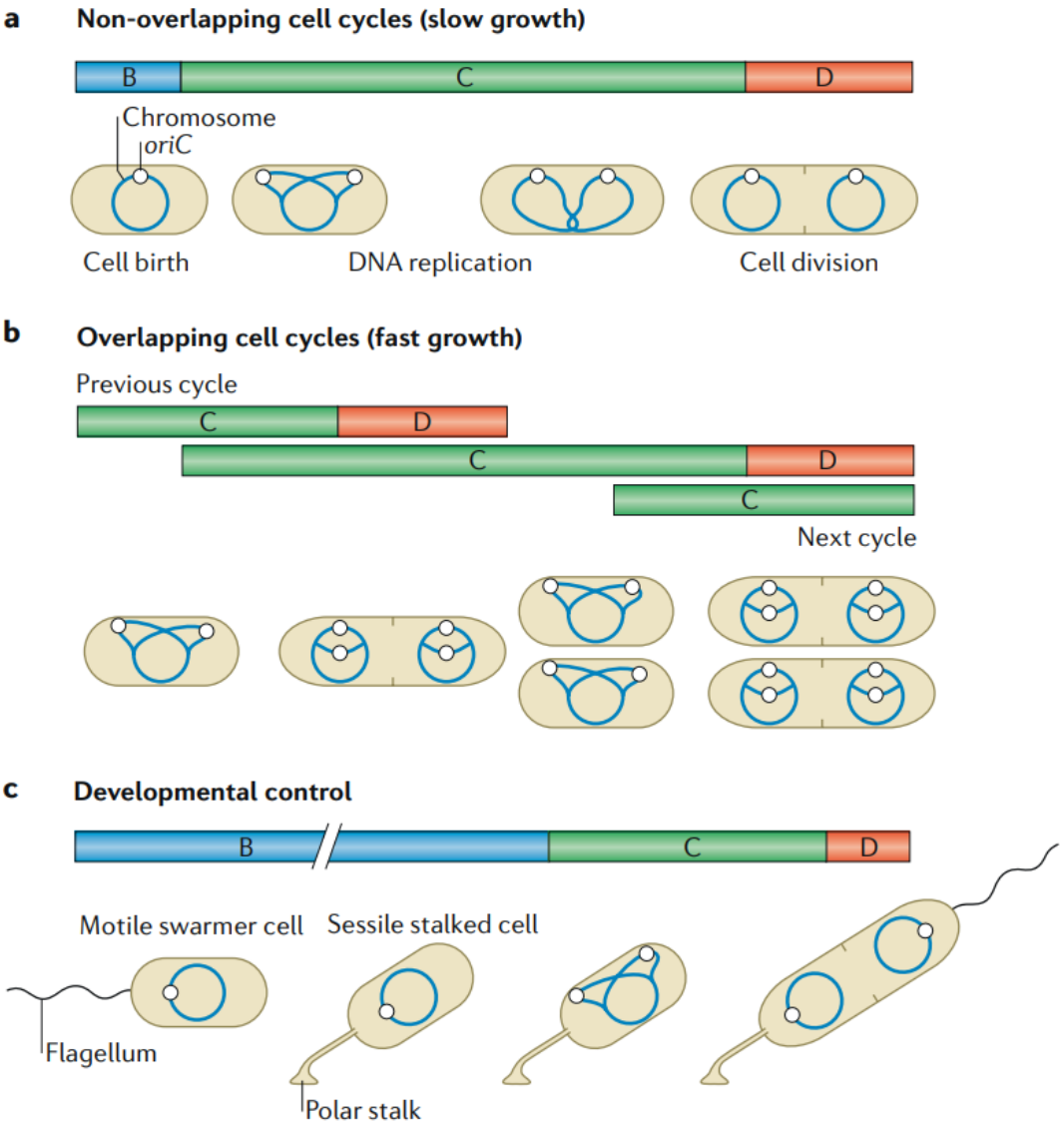


Figure 8: Schematics representing the different stages of the cell cycle in relation to growth rate.

The cell cycle is divided in three periods. The period from birth to the initiation of DNA replication is termed B and represented by a blue rectangle in the scheme, a period comprising the replication of DNA is termed C and represented by a green rectangle and the period from DNA replication completion to cell division is termed D. a) For slow-growing bacteria, all the periods are distinct, the generation time is longer than the combined C and D periods. b) In fast-growing bacteria, the B period is absent and the generation time is shorter than the combined C and D periods. As a consequence, multiple DNA replication initiation can take place at the same time. c) In the case of a developmental control such as in *Caulobacter crescentus*, only the cells differentiated into sessile stalked cells are able to replicate their DNA (Reyes-Lamothe and Sherratt, 2019).

Accordingly, the bacterial cell cycle is highly regulated by external stimuli, especially nutrient limitations. Most bacteria respond to starvation by entering a developmental cycle consisting in cell differentiation, for example; sporulation in *B. subtilis*, fruiting bodies in *Myxococcus* aerial mycelium in *Streptomyces* or bacteroids in *Sinorhizobium* during symbiosis with legume plants (that will be detailed in the next sections). Nonetheless, the slow-growing alphaproteobacterium *C. crescentus* is particularly interesting. Indeed, independently of nutrient availability or external conditions, the bacteria undergo a cell differentiation during growth, this morphogenic changes are mandatory to go further into its cell cycle. Indeed, *C. crescentus* produces two daughter cells that are morphologically and functionally different; a stalked cell, which enters a new cell cycle immediately after cell division completion, and a smaller motile non-replicative cell that requires a differentiation into a stalked cell to start a new cell cycle. Thus, similarly to eukaryotes, different cell cycle phases are distinguishable (G1, S, and G2, equivalent to B, C and D, respectively) (Figure 9). Moreover, as in eukaryotes the cell cycle regulation of *C. crescentus* is based on cyclin-dependent kinase inhibitor-like protein, CtrA (for cell cycle transcriptional regulator A, see next sections for more details), that intervenes in oscillatory manner triggering the entry into the G1-phase, thus, acting as checkpoints for the different cell cycle phases. Hence, the study of the bacterial cell cycle is essential in our understanding of this biological process in general and in the comprehension of the eukaryotic cell cycle regulation (Figure 9) (Amon, 1998).

4- The alphaproteobacterial cell cycle

The class of *Alphaproteobacteria* regroups a diversity of bacteria that are characterized by a variety of lifestyles, including free-living bacteria (*C. crescentus*), plant symbionts (rhizobia; *S. meliloti)* and pathogens (*Agrobacterium*), pathogens of animals (*Brucella, Rickettsia* and *Bartonella*). This bacterial subdivision represents a breeding ground for cell cycle models especially *C. crescentus*, even if notable differences can be observed between these bacteria due to their various lifestyles, the main logic of the cell cycle architecture is maintained (Brilli *et al.*, 2010).

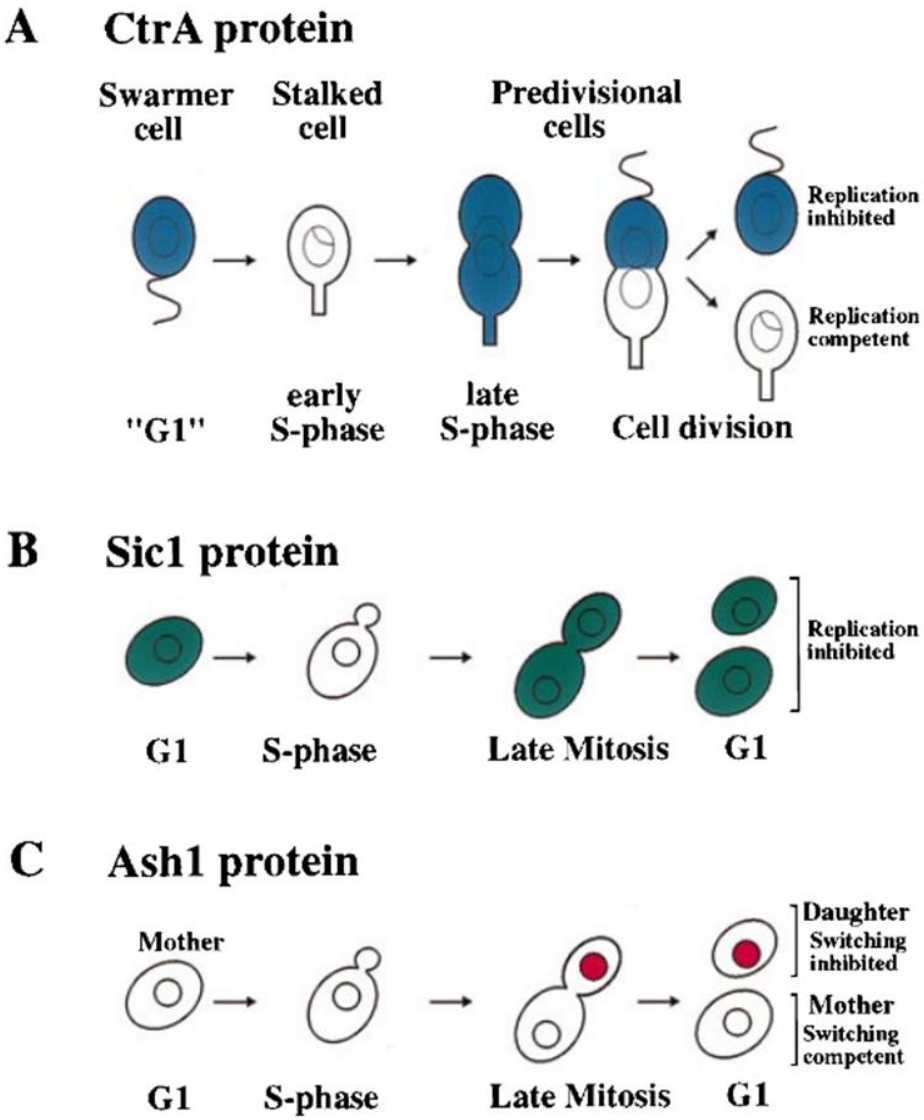


Figure 9: Comparison of *Caulobacter crescentus* and *Saccharomyces cerevisiae* cell cycle and associated regulators.

Sic1 and Ash1 are proteins implicated in the G1-phase of S. *cerevisiae,* (CDK inhibitor and HO endonuclease inhibitor respectively). A) The predivisional cell divides asymmetrically to yield swarmer and stalked daughter cells. The stalked cell enters immediately a new cell cycle while the swarmer cell has to differentiate into a stalked one to initiate a new cell cycle. CtrA accumulates in G1-phase and the late S-phase to allow cell division. B) Same behavior for Sic1 protein that accumulates at the G1-phase and is degraded at the S-phase to permit the DNA replication. C) Ash1 accumulates asymmetrically in the daughter cell, similarly to CtrA thus preventing mating-type switching (Amon, 1998).

C. crescentus was first isolated in a tap water in Boston; this bacterium became a pioneer in the cell differentiation and asymmetrical cell division studies. More interestingly about this bacterial model is that *C. crescentus* replicates its DNA only once per cell cycle (Figures 8,9). This feature allows, among other physiological traits, the synchronization of *C. crescentus* culture making it possible to study the cell cycle phases separately and at the single cell level. The synchronization procedure consists of isolating the swarmer cells by density centrifugation this latter are introduced in a fresh rich medium to start a new cell cycle in synchronized manner (Barrows and Goley, 2023).

Because of its importance, the cell cycle is a highly regulated process that involves many factors (for example the master regulators DnaA, CtrA, GcrA, CcrM and their associated regulators), all embedded to each other thus resulting in very complex regulation network (Collier, 2012), which will be explained in more details in the next paragraph (Figure 10). It was demonstrated that the expression of these factors fluctuates during the cell cycle progression. Indeed, more in general, 553 genes (19% of the genome) transcripts varied during the *C. crescentus* cell cycle, implying that these factors are expressed only when their function is needed (Laub *et al*., 2002).

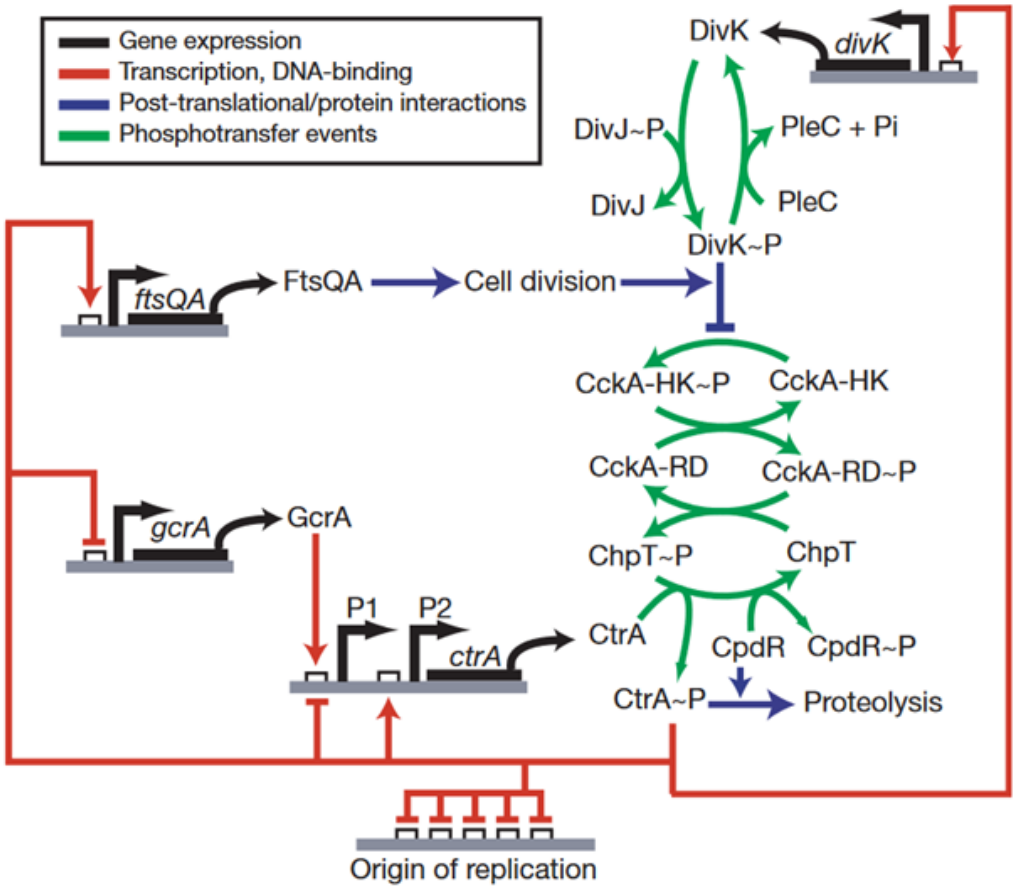


Figure 10: Diagram representing the genetic network controlling cell cycle progression in *Caulobacter crescentus.*

Biochemical relationships between components are color-coded as indicated in the key (Biondi *et al.*, 2006).

5- The cell cycle in *Sinorhizobium meliloti*

A part of the following section was published as a book chapter in Cell Cycle Regulation and Development in alphaproteobacteria, Springer International Publishing (Dendene et al., 2022)

S. meliloti is a Gram-negative soil bacterium that belong to the class *Alphaproteobacteria*. This bacterium is able to establish a symbiotic interaction within symbiotic organs, called nodules, of legumes plants such as *Medicago sativa*. There, it fixes the

atmospheric nitrogen into ammonia and obtains in return carbon sources and other nutrients. This bacterium possesses three large replicons: a *ca*. 4 mega-bases circular chromosome with a single DnaA-dependent origin of replication, a second smaller replicon (1.9 Mbp), named pSymB, that contains several essential genes and many genes involved in the adaptation to environmental niches and finally a dispensable smaller megaplasmid (1,5 Mbp), named pSymA, carrying genes mostly associated to symbiosis (Galibert *et al.*, 2001; Capela *et al.*, 2001; Finan *et al.*, 2001) (Figure 11). Surprisingly, the initiation of replication of the three origins of replication is temporally and spatially separated in the cell, with the chromosome being the first to be replicated with its origin located very close to the polar regions of the cell. The megaplasmid pSymB follows the chromosome replication with its origin located in proximity of the pole but shifted towards the center of the cell. Finally, pSymA replication starts after pSymB and its origin at the beginning of its replication is localized almost at mid-cell (Frage *et al.*, 2016). This remarkable spatial and temporal organization suggests that DNA replication in *S. meliloti* is highly organized by mechanisms that are still unknown.

S. meliloti division is asymmetrical and always produces two different cell types, a "small" type and a "large" type (Figure 11). The large cell is able to replicate its genome and produce new small and large cells. The small cell, on the contrary, has not the capacity to replicate its DNA and divide immediately. It must first differentiate into a large cell before initiating a new cycle (De Nisco *et al.*, 2014). This morphological asymmetry imposes thus a continuous asynchrony between the subsequent cell cycles of the daughter cells after division. To our knowledge, there is no exception in *S. meliloti* to the rule of one single round of genome replication per cell division as the origin of replication is strictly controlled by multiple regulatory mechanisms that ensure this perfect coordination between DNA replication and cell division (De Nisco *et al.*, 2014; Pini *et al.*, 2015).

The expression of almost 500 genes varies as a function of cell cycle in *S. meliloti*, and these genes show peak expression corresponding to the timing of their cellular function (De Nisco *et al.*, 2014). This time-regulated expression of genes, which are required for specific functions, was analyzed by synchronization of *S. meliloti* culture. Since the method developed for *C. crescentus* is not applicable, due to the slighter asymmetry of *S. meliloti*, a new synchronization method was developed based on the induction of the stringent response (induced by carbon and nitrogen starvation) able to induce G1-blocked cells by Rel-dependent ppGpp accumulation (De Nisco *et al.*, 2014). G1-blocked cells were then able to proceed through a complete and synchronized cell cycle with only one DNA replication cycle,

ultimately leading to an asymmetrical cell division. DNA replication was analyzed further by tracking the origin of replication of the different replicons of *S. meliloti* (Frage *et al.*, 2016).

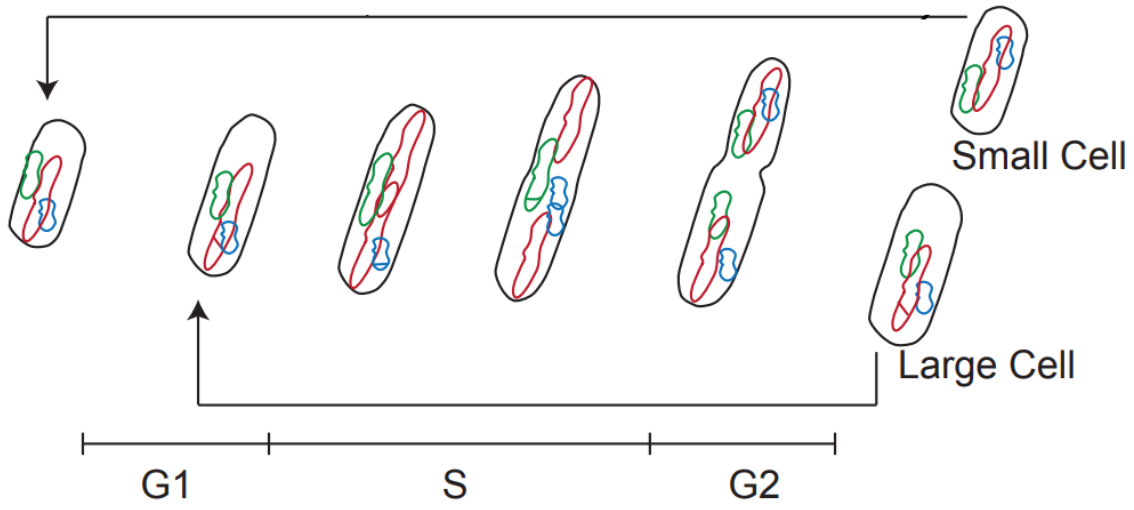


Figure 11: Scheme representing the *Sinorhizobium meliloti* cell cycle.

S. meliloti bacterium is rod-shaped and contains three replicons, here represented with three different colors. The chromosome, in red, the chromid pSymB in green, and the smallest replicon the mega plasmid pSymA in blue. Every cell division produces two different cell types: a large cell and a small cell, each containing a copy of each replicon. The large cell is able to immediately initiate a new round of DNA replication (S-phase), while the small cell (G1-phase) must first differentiate into a large cell to start a new cell cycle (Xue and Biondi, 2019)

Cell cycle regulation in *Sinorhizobium meliloti*

As revealed by the bioinformatic analysis of alphaproteobacterial genomes, almost all factors that regulate the cell cycle in the model system *C. crescentus*, are also present in *S. meliloti* (Brilli *et al.*, 2010). The conservation obviously suggests the evolution of the cell cycle program in a common ancestor of the two organisms. However, as we will specifically discuss here for *S. meliloti*, every alphaproteobacterial species appears different from the others by displaying variations on the common theme, suggesting that the cell cycle machinery has subsequently diverged in order to adapt to different lifestyles and physiologies. The adaptation to intracellular life or life in host tissues in the case of alphaproteobacterial species interacting

with eukaryotes (rhizobia, *Brucella*, *Agrobacterium* etc.) involves the formation of specific infecting cell types that may have required the evolution of particular cell cycle regulators.

Regulation of cell cycle in *S. meliloti* and other alphaproteobacterial species is based on a small number of conserved master regulators of the cell cycle. These master regulators coordinate most of the genes controlling essential steps in cell cycle progression and together constitute the master regulatory circuit of the cell cycle. Although our knowledge is still preliminary in many bacterial models, it is reasonable to assume that the master regulators DnaA, GcrA, CtrA and CcrM are well-conserved cell cycle factors in most of the species of the class *Alphaproteobacteria* (Wright *et al.*, 1997; Barnett *et al.*, 2001; Brilli *et al.*, 2010). The four master regulators, at least in *C. crescentus,* where they have been studied since the early 1990's, are synthesized in succession to drive sequential steps of the cell cycle but also to directly activate the downstream master regulator (Figures 10,12). DnaA activates *gcrA*, GcrA activates *ctrA*, CtrA activates *ccrM*, and finally, the DNA methylase CcrM resets the cycle by completely methylating the genome. DnaA, as mentioned before, is a protein that activates the initiation of DNA replication in bacteria (Sibley *et al.*, 2006; Skarstad and Katayama, 2013). However, DnaA has a dual role and is also involved in the transcriptional regulation of the next master regulator gene *gcrA* although the mechanism by which DnaA is able to activate gene expression remains still elusive. GcrA promotes on its turn *ctrA* transcription, most likely indirectly since it is probably not a transcription factor. CtrA (Cell cycle Transcriptional Regulator A) is a DNA-binding response regulator, member of the two-component signal transduction family. CtrA is the most interconnected regulator of the four master regulators. CtrA controls transcription of the downstream master regulator *ccrM* but it also inhibits *gcrA* transcription, promotes its own transcription and inhibits DnaA-mediated DNA replication through binding of the replication origin. Finally, the methylase CcrM exerts a negative epigenetic regulation on its own expression and expression of *ctrA* and a positive epigenetic regulation on *dnaA* expression. In addition, it methylates the origin of replication possibly making it competent for a new round of replication, although its precise role with respect to the initiation of DNA replication is not yet uncovered. A second regulatory circuit is integrated in this loop of master regulators at the level of CtrA and is discussed in detail in the following paragraph.

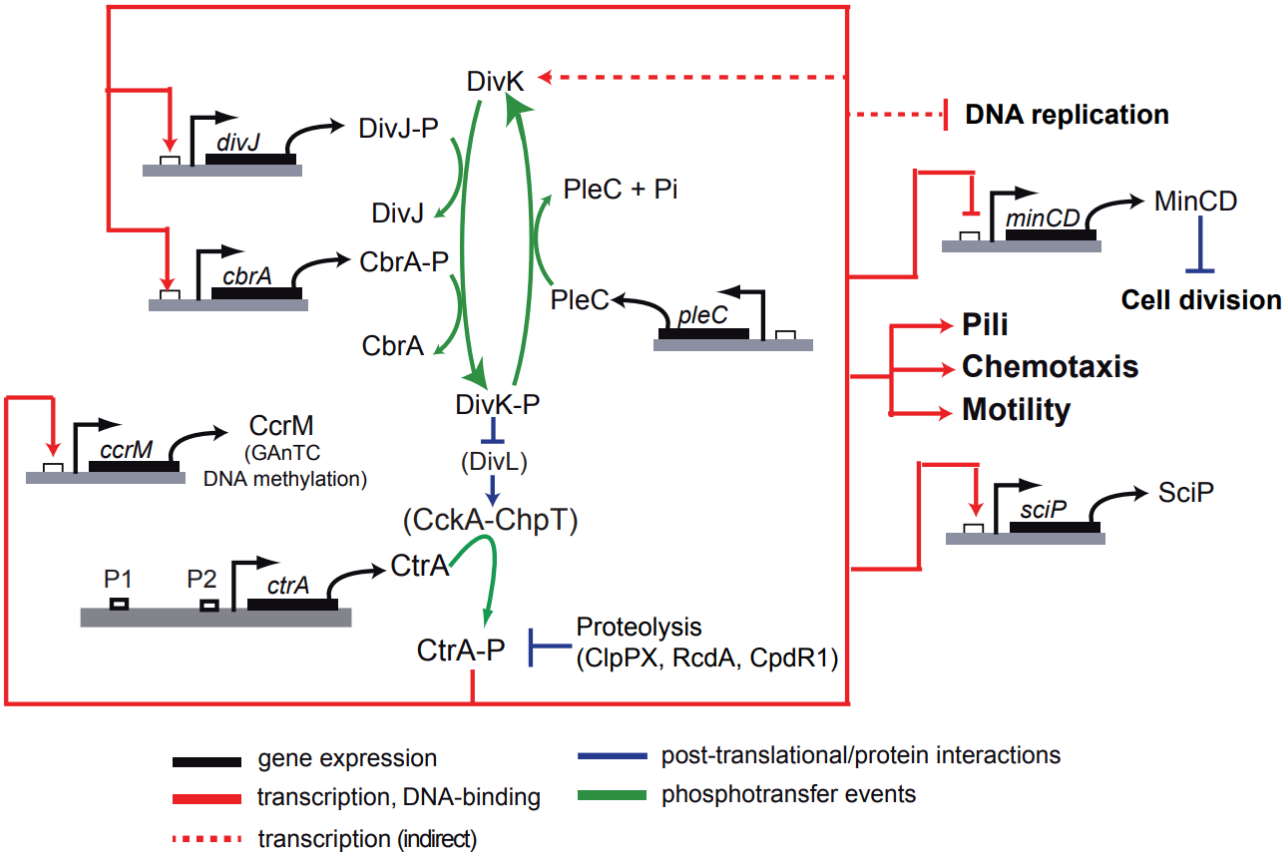


Figure 12: The cell cycle regulation network in *Sinorhizobium meliloti*. Biochemical relationships between components are color-coded as indicated in the key (Xue and Biondi, 2019).

CtrA can be considered as the most important cell cycle regulator in *S. meliloti*. The crucial role in the regulation of the cell cycle by the CtrA response regulator was demonstrated for the first time in the model species *C. crescentus* (Quon *et al.*, 1996). Phosphorylation of the REC domain leads to dimerization (Gao and Stock, 2009), creating an active dimer of the response regulator that is able to bind its consensus sequence (a palindromic sequence composed by two half sites) located in the promoter region of target genes and activate their expression. CtrA presumably belongs to this class of response regulators suggesting that a dimeric form of phosphorylated CtrA interacts with its palindromic consensus sequence that we can approximate to the sequence AATT(N_7)AATT. This consensus sequence is conserved across alphaproteobacterial genera, spanning from *Rickettsia* to *Caulobacter*, *Sinorhizobium*, *Magnetospirillum* or *Rhodobacter* (Brassinga *et al.*, 2002; Brilli *et al.*, 2010; Mercer *et al.*, 2010; Greene *et al.*, 2012). Based on the presence of this consensus in the promoter region of

genes of alphaproteobacterial genomes, the conservation of functions in this bacterial class was analyzed *in silico*, revealing that regulation by CtrA is usually linked to motility, which is probably the ancestral function controlled by CtrA (Brilli *et al.*, 2010; Greene *et al.*, 2012; Mercer *et al.*, 2012). In species belonging to the *Caulobacterales* (including *C. crescentus*) and *Rhizobiales* (*S. meliloti*, *B. abortus* and *A. tumefaciens*, for example), CtrA controls, in addition to motility, cell cycle-related functions (Brilli *et al.*, 2010). This recruitment of CtrA to the essential function of cell division is obviously associated to the essentiality of the gene *ctrA* in these species (Quon *et al*. 1996; Barnett *et al*. 2001; Pini *et al*. 2015). Conversely, in species in which CtrA controls only motility, CtrA is not essential for bacterial viability and its disruption only affects the flagellum biogenesis and possibly some other non-essential functions (Greene *et al.*, 2012a; Mercer *et al.*, 2012).

In cell cycle regulation, CtrA controls DNA replication and cell division. Genome replication is affected in a negative way. In *C. crescentus*, this inhibition is direct by binding of phosphorylated CtrA (CtrA~P) to several CtrA boxes present in the origin of replication, preventing DnaA to initiate replication (Quon *et al.*, 1998) (Figure 10). In contrast, in *S. meliloti* this control cannot be direct as there are no CtrA boxes in its origin of replication (Sibley *et al.*, 2006; Pini *et al.*, 2015) (Figure 12). However, upon depletion of CtrA, the cell fails to block the reinitiation of DNA replication, resulting in cells with multiple DNA copies. This observation suggests that also in *S. meliloti* some CtrA-dependent mechanisms for inhibition of DNA replication exist. Thus, even if the molecular mechanisms are different between *C. crescentus* and *S. meliloti*, in both strains the function of CtrA to inhibit replication is conserved. Cell division on the contrary, is positively regulated by CtrA (Quon *et al.*, 1996; Pini *et al.*, 2015a). Although the gene sets regulated by CtrA are strikingly different in alphaproteobacteria, among the conserved ones there are those involved in motility and chemotaxis functions, DNA methylation and cell division (Laub *et al.*, 2002; De Nisco *et al.*, 2014; Pini *et al.*, 2015). For example, in *S. meliloti*, CtrA represses the Min system, which inhibits septum formation and division by preventing FtsZ polymerization and Z-ring formation (Figure 12), while in *C. crescentus*, which lacks the Min system, *ftsQA* transcription is positively regulated by CtrA (Figure 10). The dual and opposite activity on replication and division places CtrA at the center of the strict cell cycle control in alphaproteobacteria. It further suggests that CtrA levels must change during the cell cycle: at the onset of DNA replication, CtrA must be inactive in order to activate DNA replication, while in the pre-divisional step

CtrA must be present in order to activate crucial division functions. This observation implies that CtrA activity must be highly regulated (Tsokos and Laub, 2012).

To ensure the oscillatory expression and activation of CtrA in the cell, this latter is regulated at the post-translational level through phosphorylation and proteolysis. Indeed, CtrA has to be dephosphorylated and degraded to promote the DNA replication. As in eukaryotes, compartmentation plays an important role in CtrA regulation. Thus, polarly localized histidine kinases determine the faith of the progeny by phosphorylating or dephosphorylating CtrA (Tsokos and Laub, 2012).

As previously mentioned, the active form of CtrA is phosphorylated. In *C. crescentus*, this phosphorylation is orchestrated by a fine-tuned phosphorelay that can be divided into two core phosphorylation pathways. The first one is composed by DivJ-PleC-DivK (Figure 10). In the predivisional cell the histidine kinase DivJ is located at the stalked pole and phosphorylates the single response regulator DivK, while the bifunctional kinase PleC is located at the swarmer pole and acts as phosphatase of DivK, thus creating a gradient of DivK-P in the cell. After the completion of cell division, the DivK-P accumulates in the stalked cell while the swarmer cell is filled with the non-phosphorylated DivK. The second core phosphorylation pathway is composed by the complex; DivL-CckA-ChpT-CtrA. When DivK is not phosphorylated (in the swarmer pole), the bifunctional histidine kinase CckA is phosphorylated with the help of DivL. This factor is an atypical histidine kinase responsible of the kinase activity of CckA and its localization at the swarmer pole, the phosphate group is then transmitted from CckA to the histidine phosphotranspherase ChpT, which in turn phosphorylates CtrA. In the stalked pole, DivK is phosphorylated and binds to DivL. The absence of DivL, together with binding of c-di-GMP, synthetized by the response regulator PleD whith a cyclase activity, results in the activation of the phosphatase function of CckA and, as a consequence, the dephosphorylation/degradation of CtrA (Figure 13).

In *C. crescentus*, previous studies showed the involvement of the proteolysis in the CtrA oscillation. The degradosome complex is constituted by the ATP-dependent protease ClpXP and a group of adaptors composed of the single receiver domain CpdR, RcdA and the c-di-GMP bound PopA. These factors intervene in a coordinated and hierarchical manner to stimulate the proteolysis by bringing CtrA to ClpXP for degradation. Thus, PopA when linked to c-di-GMP, binds to CtrA and then to RcdA. RcdA and PopA are responsible for CtrA localization at the stalked cell. CpdR is also under the influence of the previously mentioned

phosphorelay (CckA-ChpT). When CpdR is dephosphorylated, it becomes active and drives the localization of the protease ClpXP to the stalked cell, the complex RcdA interacts with ClpX bringing CtrA closer to the protease (Figure 14).

All the above-cited factors are conserved in *S. meliloti*, which suggests the conservation of the same regulation network. However, differences must be noted. Based on our current knowledge, for example, *S. meliloti*, unlike *C. crescentus*, possesses, besides DivJ, a second DivK-phosphorylating histidine kinase CbrA and two copies of the single response regulator CpdR (CpdR1 and CpdR2), CpdR1 being the cell cycle regulated copy. In addition, *S. meliloti* lacks the adaptor protein PopA and unlike *C. crescentus* the c-di-GMP seems to have lost its key role in the cell cycle regulation.

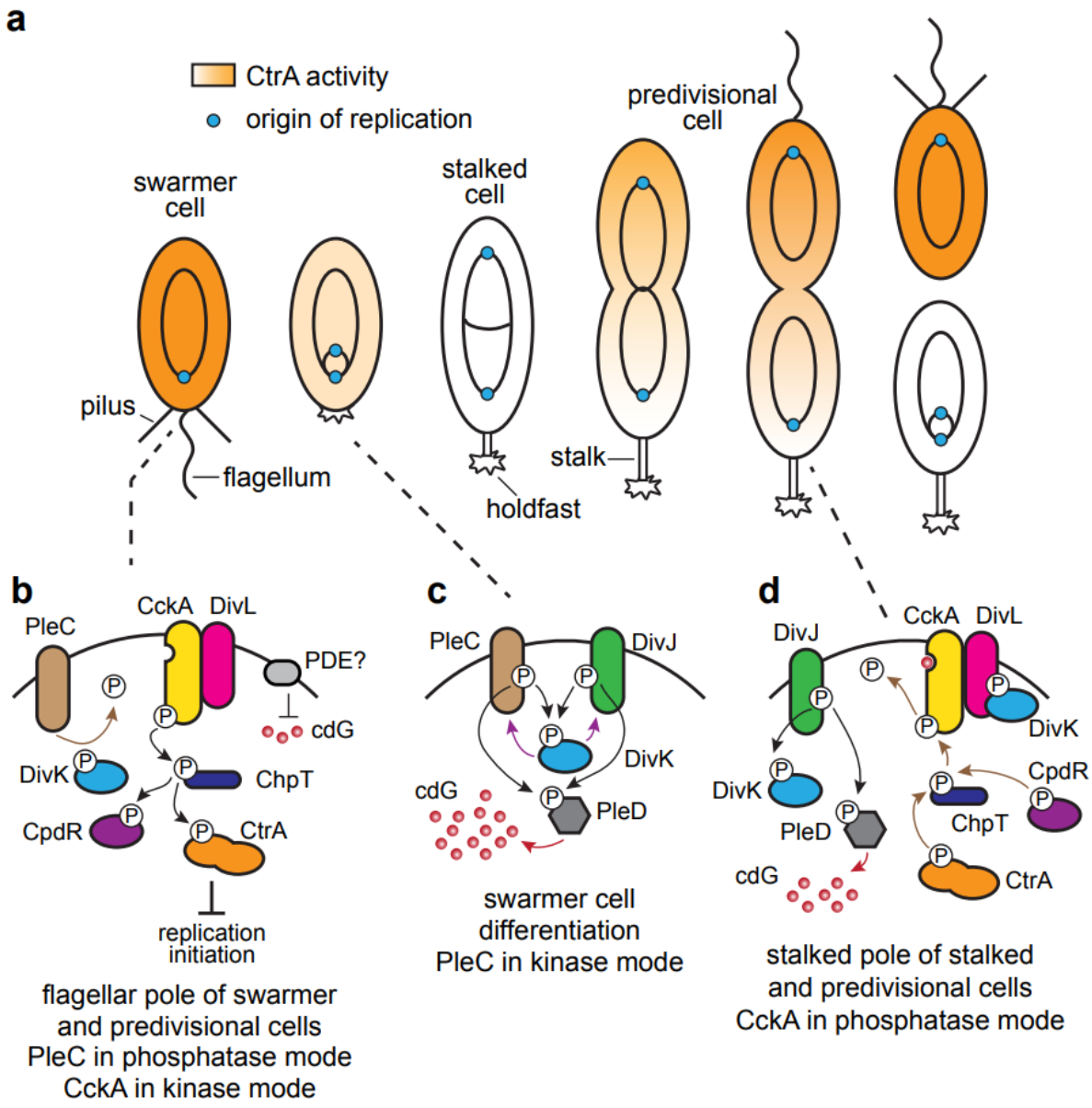


Figure 13: *Caulobacter crescentus* cell cycle is regulated by a phosphorelay complex.

a) Schematic of the cell cycle in *C. crescentus* showing the different stages of DNA replication (black ovals) and the location of the origin of replication through the cell cycle progression (blue circle). b,c,d) The phosphorelays involved in the regulation of the cell cycle and their positioning in the cell (Zik and Ryan, 2022).

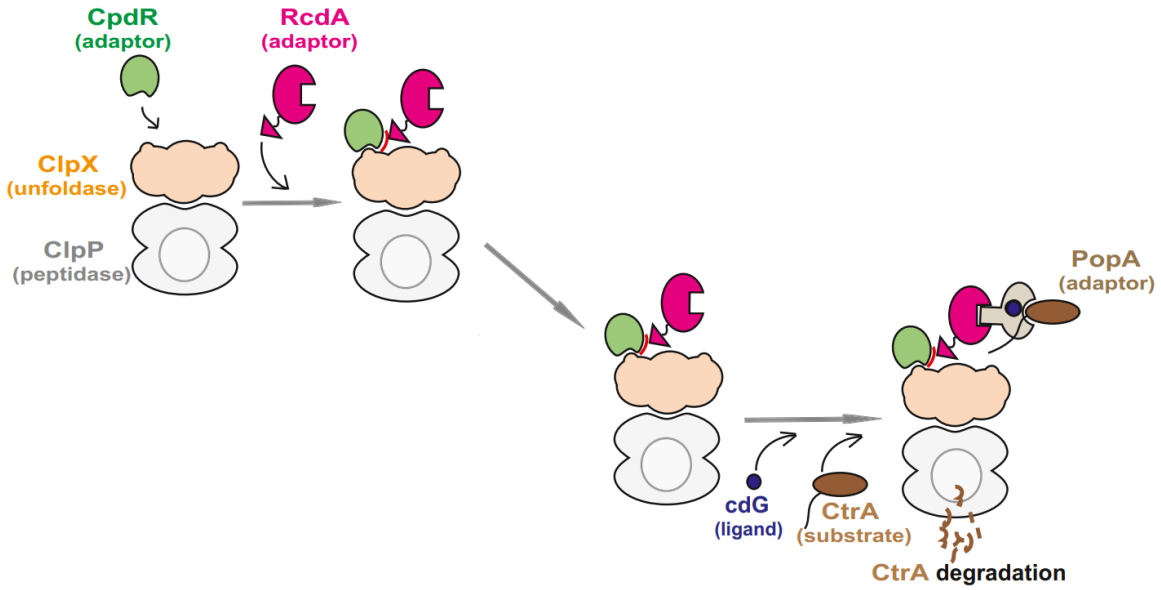


Figure 14: A hierarchal model of the proteolytic degradation of CtrA by the degradosome.

CpdR binds and localizes the protease ClpXP at the proteolysis pole. Then the CpdR-ClpXP complex recruits the adaptor RcdA. In presence of c-di-GMP (cdG), PopA delivers CtrA to RcdA, which brings CtrA closer to the protease ClpXP and allows its degradation (Mahmoud and Chien, 2018).

6- The general concept of symbiosis

In nature, the living organisms thrive wherever the conditions allow growth, in some cases some of them live inside or colonize the surface of other organisms. Indeed, microorganisms and multicellular organisms evolved in close contact, resulting in different types of interspecific associations. For instance, humans and their intestinal microbiota or mycorrhiza and plants. This cohabitation probably exists since the appearance of multicellular organisms. These relationships are of different natures. In the one hand, pathogenesis and

parasitism are interactions, where the microorganism has a negative effect on its host. In some cases, the pathogen can lead to the death of the host while the parasite can live in the infected host for the lifetime of this latter without killing it. In the other hand, symbiosis, a term introduced for the first time in 1871 by the botanist Heinrich Anton de Bary, refers to all interactions characterized by a durable relationship between organisms from different species. Among these interactions commensalism and mutualism can be distinguished (Figure 15), these interactions being beneficial to one or both interacting organisms, respectively. The symbiosis is probably implicated in the diversity of species as known today. Moreover, the most plausible evolution scenario of eukaryotes is based on the endosymbiosis phenomenon that consists in the integration of a prokaryote by an archaea as an organelle, for instance, the acquisition of an alpha-proteobacterium and a cyanobacterium that later became mitochondria and chloroplasts respectively (Gray, 2017).

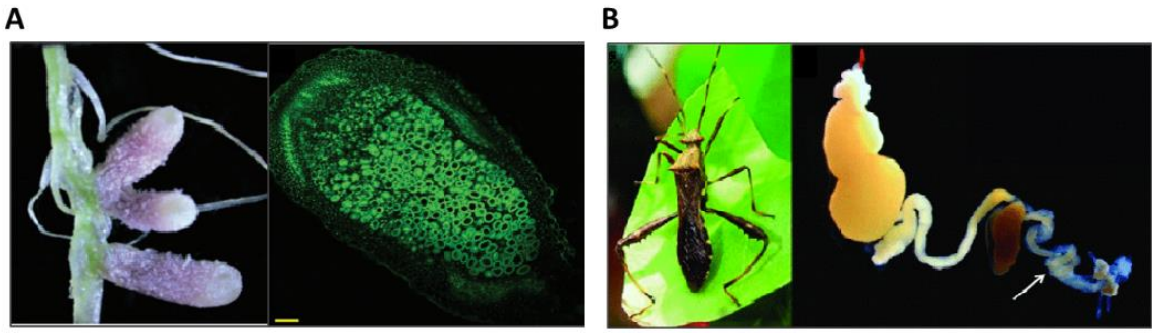


Figure 15: Examples of symbiotic relationships involving eukaryotic hosts and mutualistic microbes.

A) Nitrogen-fixing symbiosis involving the legume plant *Medicago* and the rhizobial species *Sinorhizobium meliloti*. Nodules on the plant root on the left and a confocal microscopy image of a nodule longitudinal section on the right; the bacteria appear in green (DiCenzo *et al.*, 2018). B) Gut symbiosis in the bean bug *Riptortus pedestris*. Adult male on the left and dissected midgut on the right, the midgut fourth region (M4) pointed with a white arrow represents the symbiotic region filled with symbiotic bacteria (Kikuchi *et al.*, 2012).

7- Different symbiotic relationships

Although symbiotic and pathogenic interactions have been known for more than a century, the biologists have mainly focused on the pathogenic aspect of these interactions, surely because of the emergency that it constitutes. However, the symbiosis is just as important, indeed this interaction is widely present in nature and essential for the homeostasis of many

ecosystems and this from the beginning of terrestrial environmental life. Indeed, the first land plants “Embryophytes” appeared about 470 million years ago and it is believed that this first “out of the oceans” of the plants, significantly drove the conquest of novel terrestrial environments. Moreover, these early embryophytes were characterized by a low-developed root system thus, this latter were dependent on fungal interaction, which provides a further proof of the key role and the ancestral nature of the symbiotic interactions between organisms (Dunn, 2013).

For the bacteria, an interaction with another organism may represent a solution to lighten the pressure faced due to the continuous environmental changes in free-living conditions. Indeed, the colonized host constitutes a niche with abundant nutrients for the bacteria. To do so, the bacteria have to detect the presence, be attracted and establish a physical contact with the host. Moreover, the bacteria must colonize the host without triggering its immune system. These features are shared by both symbiotic and pathogen bacteria. Interestingly, an elongated contact of the pathogen with the host two scenarios can be observed; an attenuation of the pathogen virulence, which favors a symbiotic interaction or a strengthening of the host defense (Steinert *et al.*, 2000).

8- The Nitrogen-fixing symbiosis

Nitrogen element composes most compounds that are essential for life, for instance; proteins, amino acids, nucleic acid and vitamins. The atmosphere constitutes a considerable reservoir of nitrogen. Indeed, 80% of it is composed by N_2. However, unlike oxygen, atmospheric nitrogen is not available by plants nor animals, thus, due to the high stability of the molecule ensured by the triple bond N≡N. Fortunately, some prokaryotes (archaea and bacteria) known as diazotrophs are equipped to fix the atmospheric nitrogen and they consequently contribute to the use of this essential element (Rascio and Rocca, 2008). Among them, some are free-living organisms (such as the archaea *Methanosarcina sp*. or bacteria from the Firmicutes and Bacteroidetes such as *Clostridium sp*. and *Chlorobium sp.* respectively), while other bacteria for example members of the cyanobacteria and proteobacteria are able to establish a symbiotic relationship with eukaryotes. The symbiont occupies a specialized organ or specific regions from the host where the exchange of nutrients occurs (Kneip *et al.*, 2007) (Figure 16).

There is a broad range of eukaryote hosts, for instance diazotrophic Gram-negative bacteria known as rhizobia associate with legume plants, Gram-negative bacteria of the genus *Frankia* associate with dicotyledonous plants and finally cyanobacteria, which associate with a wide spectrum of plants, protists and animals (Pawlowski and Bergman, 2007; Sepp *et al.*, 2023). The capacity of the bacteria to fix nitrogen allows the host organisms to thrive in non-optimized environments and to colonize them despite the limited availability of assimilable forms of nitrogen,

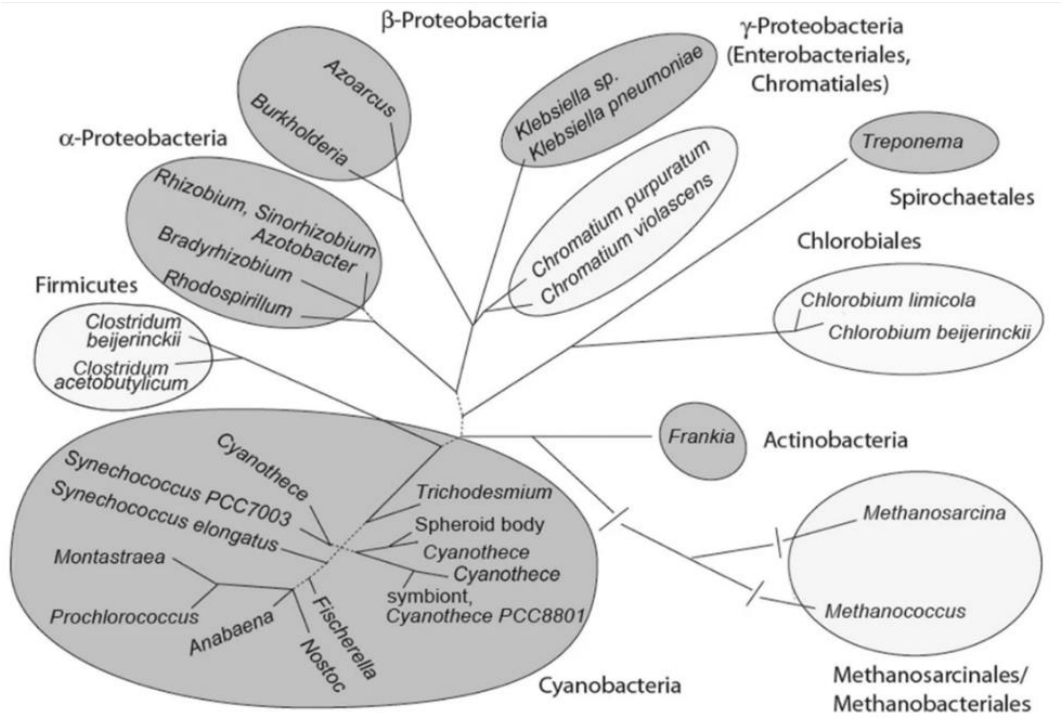


Figure 16: Phylogenetic tree of symbiotic and non-symbiotic nitrogen-fixing bacteria.

The symbiotic species are represented grey ellipses and non-symbiotic are represented in white ellipses; (classes/orders for proteobacteria). The phylogenetic analysis was realized using an alignment of the 16S rDNA genes sequences and the tree was built using PhyML (Kneip *et al.*, 2007).

a- Ecological role and importance of dinitrogen gas

Nitrogen constitutes a limiting nutrient for plant growth in agricultural systems, since plants are not able to fix atmospheric nitrogen by themselves. Moreover, with the improvement of living conditions, the human population grows exponentially and as consequence, the food needs as well. To cope with the increasing demand, big amounts of industrially produced

nitrogen are supplied to agricultural lands in order to increase the nitrogen availability in the soil and thereby to secure yields. The artificial synthesis of fertilizers is made by the Haber-Bosch process, which uses a combination of hydrogen and nitrogen under higher pressure (>100 bar) and temperature (~500°C). This reaction allows to break the triple bond of dinitrogen gas and produce ammonia. However, this reaction requires a considerable amount of hydrogen resources and fossil fuel thus representing 1 to 2% of global energy consumption (Kyriakou *et al.*, 2020). Moreover, the intensive use of fertilizers results in massive environmental pollution and health issues. The nitrate enrichment of the water caused by the leaching of nitrogen from the soil to the water leads to an acidification and an eutrophication, which significantly reduce the biodiversity (Erisman *et al.*, 2013; Tedengren, 2021). For instance, the nitrogen-fixing symbiosis that occurs between plants and microorganisms draws all attention these last decades. Indeed, it is urgent to find an alternative to fertilizers without compromising the food supply. One of the promising solutions resides in "a return to the roots" using a natural system, which is the symbiosis between legume plants and microorganisms such as bacteria. Legume plants belong to an important family of Angiosperms that regroups 19 500 species worldwide, among which all architectural types are found: herbs, shrubs, vines or trees (Figure 17). Legumes, unlike the vast majority of plants are able to establish a symbiotic relationship with microorganisms that fix atmospheric nitrogen into an assimilable form by the plant (Azani *et al.*, 2017).

Most legume plants are able to establish a nitrogen-fixing symbiosis with bacteria. During this association, bacteria are hosted in special organs called nodules. This symbiosis constitutes an ancestral trait of the family although some legume species lost their symbiotic ability. The combined nitrogen fixation activity by all legumes on earth is a key process in the biogeochemical nitrogen cycle and has therefore a tremendous impact on the ecology of our planet (Kebede, 2021). Indeed, plantation of legumes plant allows the natural enrichment of the soil with nitrogen, which prepare the soil to the plantation of other crops, for example, by a process known as "Fallow land". Legumes can also be used, as green manure to prevent the soil erosion and improve its structure (Asghar and Kataoka, 2021), or cultured with other crops in intercropping systems. This is the case of the "three sisters" (corn, beans and squash), a system practiced by indigenous people of America since centuries (Ngapo *et al.*, 2021).

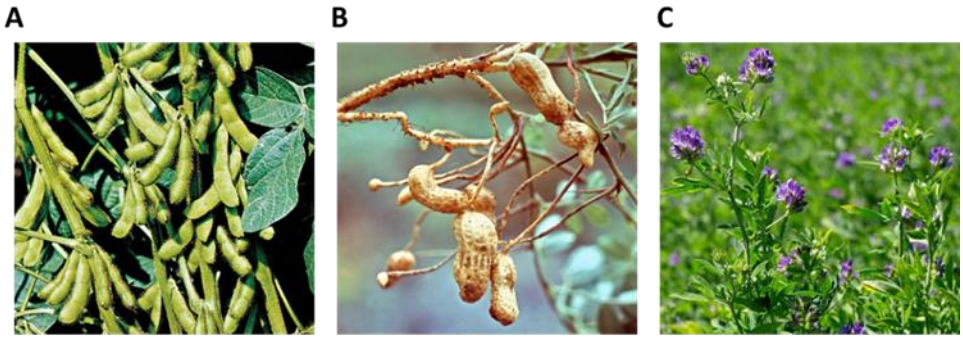


Figure 17: Examples of legumes species.

A) Image of the annual legume soybean (*Glycine max*), the economically most important pulse in the world. B) Image of a peanut plant (*Arachis hypogaea*) or groundnut. C) Image of a plant of alfalfa or lucerne (*Medicago sativa*) used as forage crop, for grazing and as a cover crop (https://www.britannica.com/plant/Fabaceae,https://www.britannica.com/plant/peanut, https://www.britannica.com/plant/alfalfa).

b- Rhizobia: to fix or not to fix

Rhizobia is a term initially referring to bacteria from the genus *Rhizobium*, those bacteria are able to establish a nitrogen-fixing symbiosis with legume plants. In 1888, Beijerinck isolated the first nitrogen-fixing bacteria from a nodule root and named it *Bacillus radicicola*, which was renamed latter on as *Rhizobium leguminosarum*. With the increasing of research in this field a larger and more diversified number of bacteria were added to the group of rhizobia. Among them, bacteria belonging to the class of *Alphaproteobacteria* (*Bradyrhizobium, Sinorhizobium, Azorhizobium, Methylobacterium, Devosia, Ochrobactrum, Pararhizobium, Neorhizobium* and *Phyllobacterium)* and bacteria from the *Betaproteobacteria* class (*Burkholderia, Ralstonia* and *Cupriavidus)* (Willems, 2006; Masson-Boivin *et al.*, 2009; Yang *et al.*, 2020) (Figure 18). Under nitrogen-limiting conditions, this interaction results in the production of a specialized symbiotic organ called nodule, where bacteria will fix the atmospheric nitrogen into ammonia.

The ability of rhizobia to fix nitrogen and to establish symbiosis is ensured by a set of genes consisting of specific molecule that detect the presence of the host symbiont and a specific enzyme that fixes the dinitrogen gas called nitrogenase respectively these elements will be described in the following paragraphs. This gene set is carried on transferrable genetic elements such as megaplasmids in *S. meliloti* and Integrative and Conjugative Elements (ICE) in

Mesorhizobium loti (Figure 18). The horizontal genetic transfer allows the spread of symbiotic traits, which can explain the polyphyletic origin of rhizobia. Indeed, the plasmid harboring the symbiotic genes can be transferred between bacteria (10^2–10^9 donor bacteria for one successful transfer). This frequency could be observed in the rhizosphere, where the density of bacteria is high, implying a role of the plant host in this genetic spread. Furthermore, some legume exudates can trigger the transfer of the symbiotic genes encoded on the ICE such as in *Azorhizobium caulinodans* (Poole *et al.*, 2018; Andrews *et al.*, 2018).

Among the rhizobium-legume interactions, the *S. meliloti-Medicago* interaction has emerged as one of the most productive model systems for the study of the nodule-forming and nitrogen-fixing symbiosis.

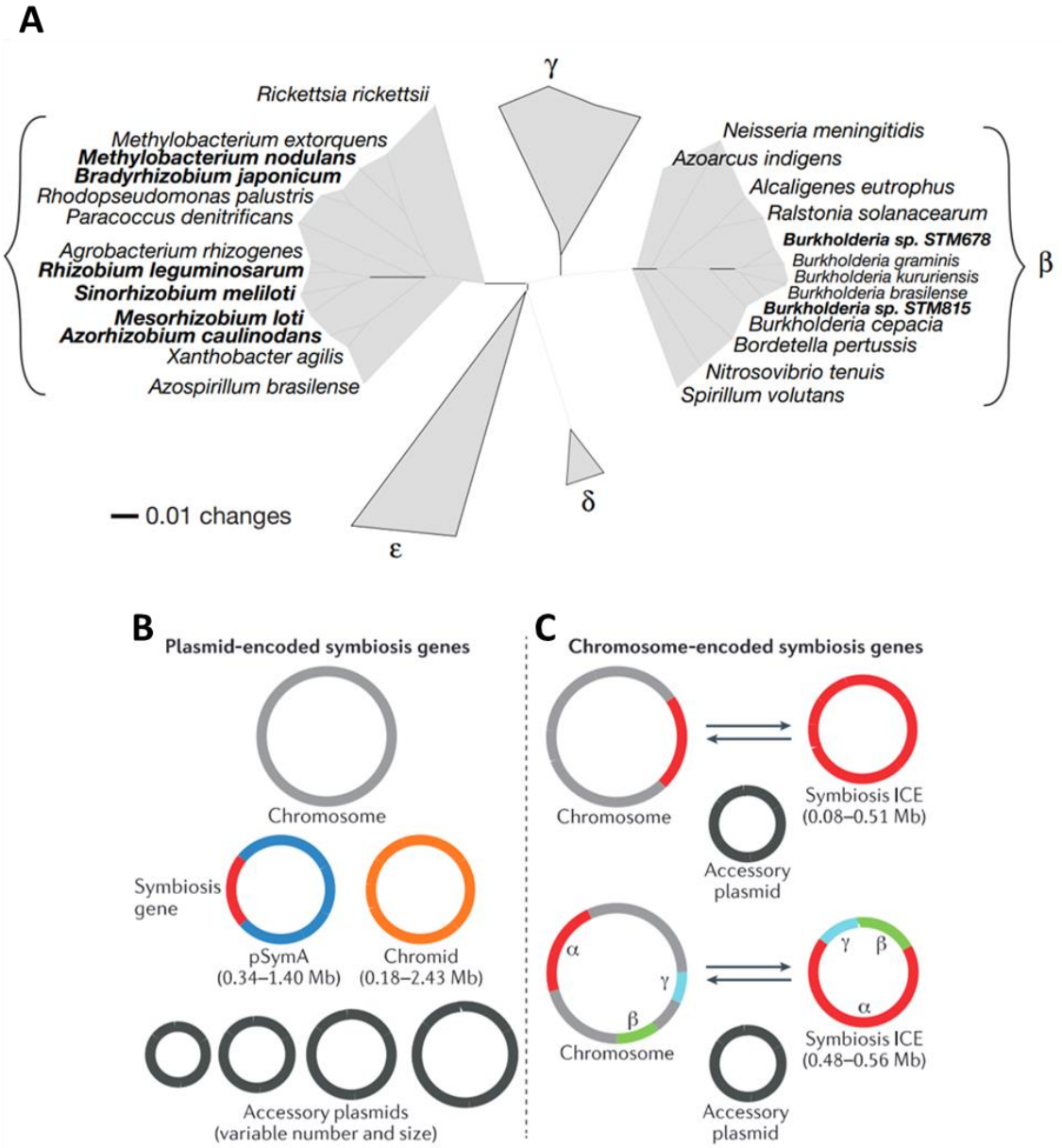


Figure 18: Phylogenetic distribution of rhizobia and genomic organization of their symbiosis genes.

A) Unrooted 16S rDNA phylogenetic tree representing the relationships between the different rhizobia. The rhizobia group includes strains from different subdivisions of the *Proteobacteria*. The tree was generated by the neighbor-joining method. The genome organization of rhizobia is usually complex consisting in a large chromosome and several amplicons such as chromids and plasmids (adapted from Moulin *et al.*, 2001). B) The symbiosis genes can be chromosomal or plasmidic. For example, in *S. meliloti* the genes are carried on a large plasmid pSymA C) in other rhizobia such as *Mesorhizobium*, *Azorhizobium* and *Bradyrhizobium*, the genes coding for symbiotic functions are carried by mobile integrative and conjugative elements (ICEs). The presence of the symbiotic genes on mobile elements is at the basis of the polyphyletic feature of symbiosis (adapted from Poole *et al.*, 2018).

9- Nitrogen-fixing symbiosis between *Sinorhizobium meliloti* and *Medicago*

S. meliloti is a free-living soil saprophyte, an endophyte and a legume endosymbiont (Poole *et al.*, 2018). The latter lifestyle has made its renown and today, it is one of the better-studied symbiotic species among all bacteria. This bacterium has the ability to colonize roots of leguminous plants of the genera *Medicago, Melilotus* and *Trigonella* (Mnasri *et al.*, 2009). As the other rhizobia, when it is in contact with plant roots, it induces the formation of nodules (Figure 19). The nodules provide to the bacteria ideal conditions to reduce atmospheric nitrogen into ammonia that is incorporated by the plant and used for its nitrogen needs to sustain growth. In exchange, the bacterium receives nutrients and an exclusive niche inside the nodules where it can establish. Starting from a single or very few bacterial cells, a very large population of several millions in a few days, the time it takes to form a mature and fully infected nodule (Jones *et al.*, 2007). Thus, unlike the soil environment, where the rate of division is low, the nodule allows the bacteria to multiply rapidly, indicating a considerable evolutionary advantage for the symbiotic lifestyle.

a- Molecular bases of symbiotic infection and differentiation

The first bacterial contact with plants consists in an exchange of specific signaling molecules. Chemotaxis, particularly towards the abundant amino acids in *Medicago* exudates, guide the *S. meliloti* cells in the soil towards the plant roots (Compton *et al.*, 2020). In absence of an assimilable nitrogen in the soil, plants will secrete flavonoids in the rhizosphere and in response to these plant molecules, rhizobia secrete lipo-chito-oligosaccharidic signals, called Nod factors (Poole *et al*. 2018). Interestingly, flavonoids have also growth-stimulating activity on the rhizobia, suggesting multiple dose-dependent ecological roles of this plant signal (Nouwen *et al.*, 2019). In the *S. meliloti – M. sativa* model, luteolin (*M. sativa*-derived flavonoid) binds to the bacterial regulator NodD1 that in turn will bind to a nod box and activate the transcription of *nodABC* and *nodIJ* genes. This set of genes code for the proteins that are required for the synthetize and the exportation of the lipo-chito-oligosaccharide Nod factors, respectively (Mergaert *et al.*, 1997; Peck *et al.*, 2006). Nod factors are recognized by the plant through membrane receptors, triggering the plant program for nodule formation and infection (Figure 19) (Oldroyd, 2013). During the nodule formation, additional bacterial molecular patterns are monitored by the plant, in particular surface polysaccharides, such as exopolysaccharides and lipopolysaccharides (Skorupska *et al.*, 2006). Together, these

molecular keys direct the symbiotic process and are highly specific, minimizing the risk of infections by non-compatible rhizobia or opportunistic and pathogenic organisms.

The entrance of *S. meliloti* in the plant tissue happens by the deformation of the normally straight growth of root hairs into a typical curled growth direction, which is specifically induced by the bacterial Nod factors. The curling root hair traps a single or very few *S. meliloti* cells, which constitute the founding cells of what will become the complete nodule population. The entrapped rhizobia are able to penetrate the root hair cell via the formation of an infection thread, a tubular structure containing dividing bacteria. The Nod factor perception in the root hair also triggers cell divisions, at a distance, in the underlying root cortical cells. These dividing plant cells form a nodule primordium that will further develop into a nodule. Simultaneously, the infection thread that was first initiated in the root hair grows and ramifies towards the primordium, thereby conducting the rhizobia towards the newly formed cells of the incipient nodule (Jones *et al.*, 2007; Oldroyd, 2013) (Figure 19).

An infection thread that has reached and penetrated a young nodule cell releases rhizobia through an endocytotic process into the plant cell. The bacteria are not released freely in the cytosol but inside vesicles, called symbiosomes, which have a plasmalemma-like membrane. Within the symbiosomes, the rhizobia grow and differentiate into their nitrogen-fixing form called the bacteroids. Repeated infections and the growth of the rhizobia in symbiosomes will ultimately result in a symbiotic nodule cell that is completely packed with intracellular bacteroids (Figure 19) (Whitehead and Day, 1997).

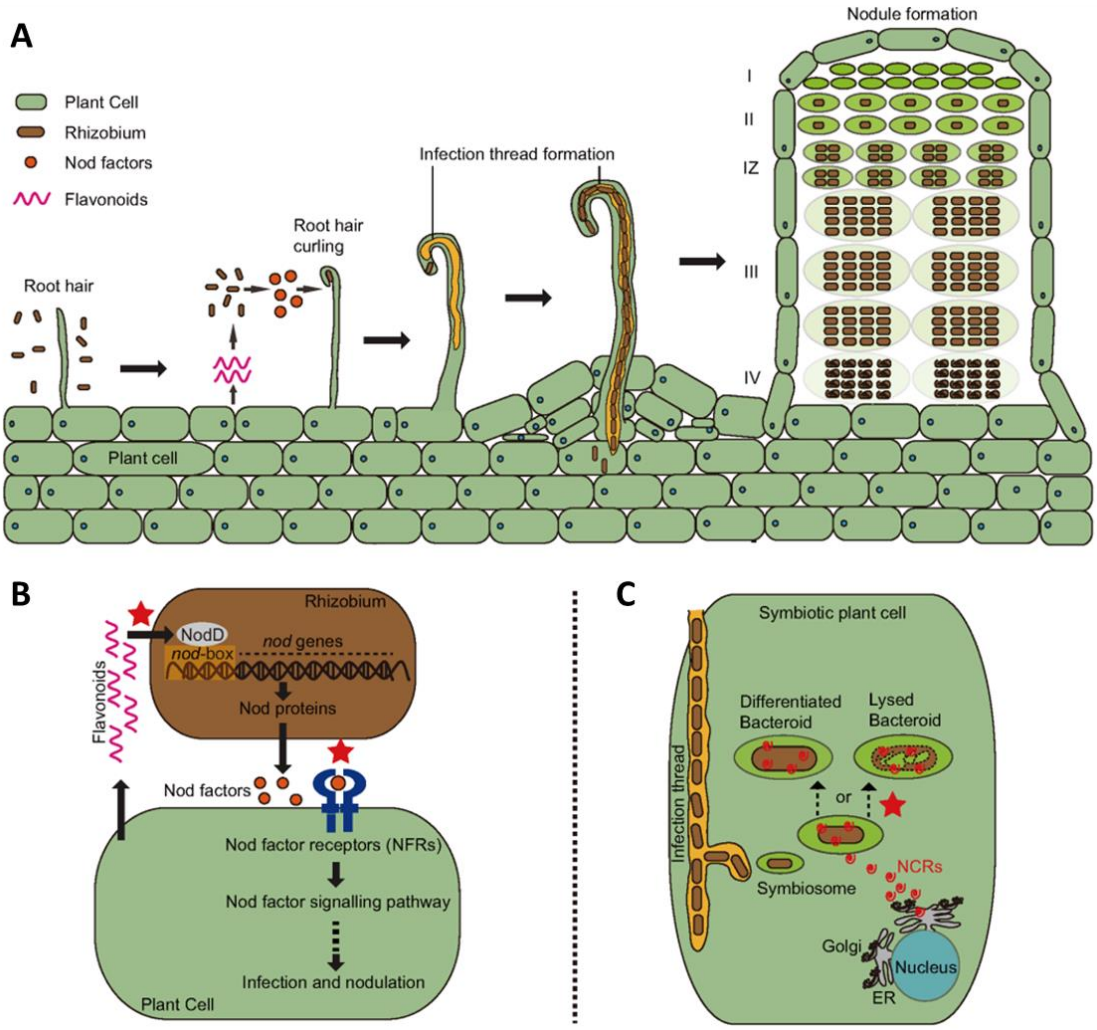


Figure 19: Scheme representing the establishment of the symbiosis between legumes and rhizobia.

A) The initiation process of the symbiosis; the legume secretes molecules (Flavonoids) that induces the expression of rhizobia Nod factors. The Nod factors (NF) are recognized by host Nod factor receptors (NFRs). This first contact induces the curling of the hair root and the formation of the infection thread, the rhizobia progress through the infection thread and are released in the nodule inside symbiosomes. The mature indeterminate nodule is composed by a meristem zone (I), an infection zone (II), an interzone (IZ), a nitrogen-fixing zone (III), and a senescent zone (IV) (B) The host secretes flavonoids, which bind to NodD proteins (transcription factors), this latter will induce the expression of *nod* genes. The enzymes coded by the *nod* genes lead to the synthesis of Nod factors (NF) that are in return recognized by the host. C) Rhizobia enter the host cell through an infection thread, each bacterium is comprised in a single symbiosome where Nodule-specific Cysteine-Rich (NCR) peptides will trigger their terminal differentiation into bacteroids. However, some non-adapted rhizobial strains cannot survive the antibacterial activity of certain NCR peptide isoforms, leading to formation of nodules defective in nitrogen fixation (Wang *et al.*, 2018b).

A plant cell that has been infected does not divide anymore but switches into a differentiation path towards a nitrogen-fixing nodule cell. This differentiation includes the activation of an endoreduplication cycle, leading to polyploidy and very strong cell enlargement as well as the activation of a specific transcriptional program that will assure the maintenance and the metabolic integration of the thousands of nitrogen-fixing endosymbionts within each individual mature symbiotic nodule cell (Mergaert *et al.*, 2020). In parallel, a few distal cells in the incipient nodule that are not penetrated by an infection thread will constitute a nodule meristem and will continue to divide. These nodules are known as indeterminate, presenting a meristem zone and different spatially distinguishable cell layers. The growth of this organ results in the elongated shape of these nodules (Figure 19). Depending of the symbiotic partners, the nodule can also be determinate, with no persistent meristem and display a spherical shape. Thus, in this latter case, the cells are homogeneous, since the growth is sequential and takes place at once. The determinate nodules can be found for example in the legumes *Glycine* (soybean), *Lotus* (bird's-foot trefoil), *Phaseolus* (common bean) or *Vigna* (mung bean).

First of all, the bacteroid formation implies a switch in the bacterial physiology that is adapted to the nitrogen fixation process. This switch is made possible by complete remodulation of the free living physiological program and a massive transcriptional activation of a large set of genes encoding nitrogen fixation and associated respiratory functions, which are completely silent in the free-living state (Roux *et al.*, 2014). Indeed, in the infected cell, low oxygen concentration is observed. This microaerobic environment is sensed by the regulatory cascade, composed of the FixLJ two-component regulator, and the downstream NifA and FixK transcription factors (Bobik *et al.*, 2006). In low oxygen concentration, the histidine kinase FixL is phosphorylated, this phosphate is then transferred to the response regulator FixJ, which in turn will activate the expression of several genes, among them the transcriptional regulators *fixK* and *nifA.* FixK activates the expression of *fixNOPQ,* genes coding for the cytochrome cbb3-type oxidase implicated in the respiratory chain, and NifA activates the transcription of *nifHDK* coding for the nitrogenase complex (Terpolilli *et al.*, 2012) (Figure 20). One feature of this nitrogenase is its sensitivity to the oxygen. Thus, the maintenance of a low oxygen concentration in the infected cells is a requirement, and this is made possible by the involvement of a protein called the leghemoglobin, that buffers the oxygen in order to ensure a low concentration that able the bacteroid respiration but at the same time avoid the inactivation of the leghemoglobin (Ott *et al.*, 2005).

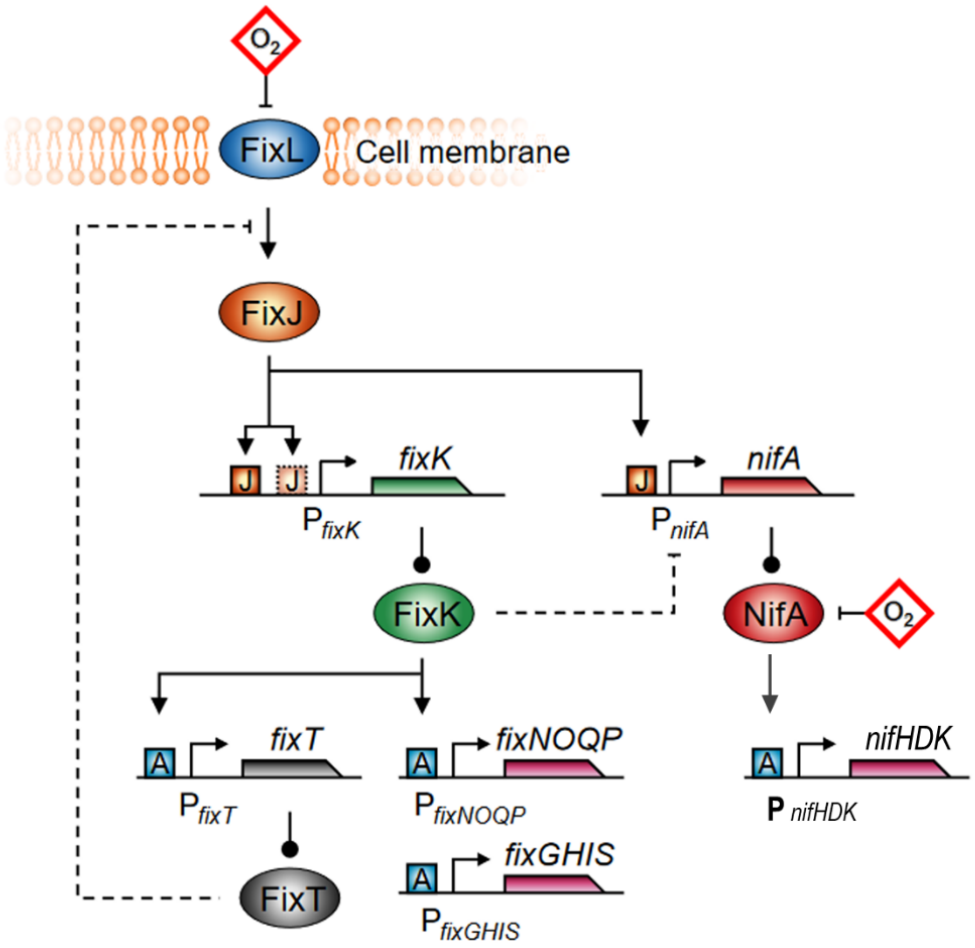


Figure 20: Scheme representing the nitrogen fixation gene network regulated by the oxygen in *Sinorhizobium meliloti.*

When the oxygen concentration is low, the two-component system FixLJ activates the transcription of FixK, which in turn activates the expression of the *fix* operon and *nifA*. NifA, which is sensitive to the oxygen, activates the expression of the genes coding for the nitrogenase enzyme (Terpolilli *et al*., 2012; Rutten and Poole, 2019).

b- The terminal differentiation of the symbiont

During the establishment of the symbiotic organ, *S. meliloti* infects symbiotic plant cells and become intracellular nitrogen-fixing organelle-like structures, called bacteroids. The bacteroids undergo a drastic differentiation program, resulting in cells that are unable to divide and produce offspring, therefore in a terminally differentiated state cells, which is in various ways dramatically different from its free-living state in the soil (Figure 21). This state of *S.*

meliloti is characterized by the irreversible loss of capacity to resume growth and to reproduce. The bacteroids also have a partially permeabilized membrane (Mergaert *et al.*, 2006).

The terminal bacteroid differentiation is a process that is determined by the host rather than being uniquely encoded in the genetic repertoire of the rhizobia. Indeed, terminal differentiation is not happening in all legumes. It is for example taking place in the Inverted Repeat Lacking Clade (IRLC) and Dalbergioid clade to which respectively *Medicago* and *Aeschynomene* genera belong but it is not happening in the Robinioid or Millettioid clades containing the well-studied *Lotus* and *Glycine* genera, respectively (Mergaert *et al.*, 2006; Czernic *et al.*, 2015). Broad host range rhizobia or engineered strains that have a switched host range, will form terminally differentiated bacteroids or not according to the host species in which they are found. This suggests that the terminal differentiation is in the first place determined by the plant, although also the bacterial genetic repertoire contributes to the extent of the bacteroid differentiation process (Mergaert *et al.*, 2006; Nicoud *et al.*, 2020).

Based on a phylogenetic analysis of the bacteroid state in the legume family, it was proposed that the ancestral state of bacteroids is the undifferentiated type, the type that is found in the Robinioids or Millettioids (Oono *et al.*, 2011). According to this scenario, terminal bacteroid differentiation has evolved several times in the legumes and appeared independently in for example the IRLC and Dalbergoid legumes.

From an evolutionary point of view, this terminal differentiation is puzzling at first sight, because the absence of offspring seems to be incompatible with the natural selection of this process. However, since the nodule bacteria form a clonal or nearly clonal population, even if the majority of them is terminally differentiated, still a relatively large population of genetically identical undifferentiated bacteria remain in the nodules and those can replenish the soil rhizobia from senescing nodules. However, it still seems to be difficult to explain why a bacterium, in the context of a beneficial symbiosis, sacrifices billions of siblings for the cause of the plant. At its most basic level, the terminal differentiation of *S. meliloti* corresponds to a transformation of its regular cell cycle into an endoreduplication cycle with no offspring generation.

c- The NCR peptides, host effectors of bacteroid differentiation

The identification of the host factors that trigger the terminal bacteroid differentiation was based on genomic and transcriptomic comparisons of legumes that display the feature or not (Mergaert *et al.*, 2003; Alunni *et al.*, 2007; Van de Velde *et al.*, 2010). These analyses correlated the formation of terminally differentiated bacteroids with the expression in nodules of a particular family of peptide-encoding genes, which were called the NCRs for nodule-specific cysteine-rich peptides (Figure 21). In *M. truncatula*, these peptides are specifically produced in nodules, in the infected symbiotic cells, and nowhere else in the plant (Guefrachi *et al.*, 2014). Remarkably, many legume species produce a large diversity of them, sometimes over several hundred different ones. For example, *M. truncatula* expresses over 600 different *NCR* genes in nodules (Montiel *et al.*, 2017). NCR peptides are small secretory peptides characterized by a pattern of conserved cysteine residues (Figure 21C). Importantly, the NCRs are related to antimicrobial peptides, which are innate immunity effectors that are used by eukaryotic hosts, namely plants and animals, to attack and eliminate invading microbes (Mergaert, 2018).

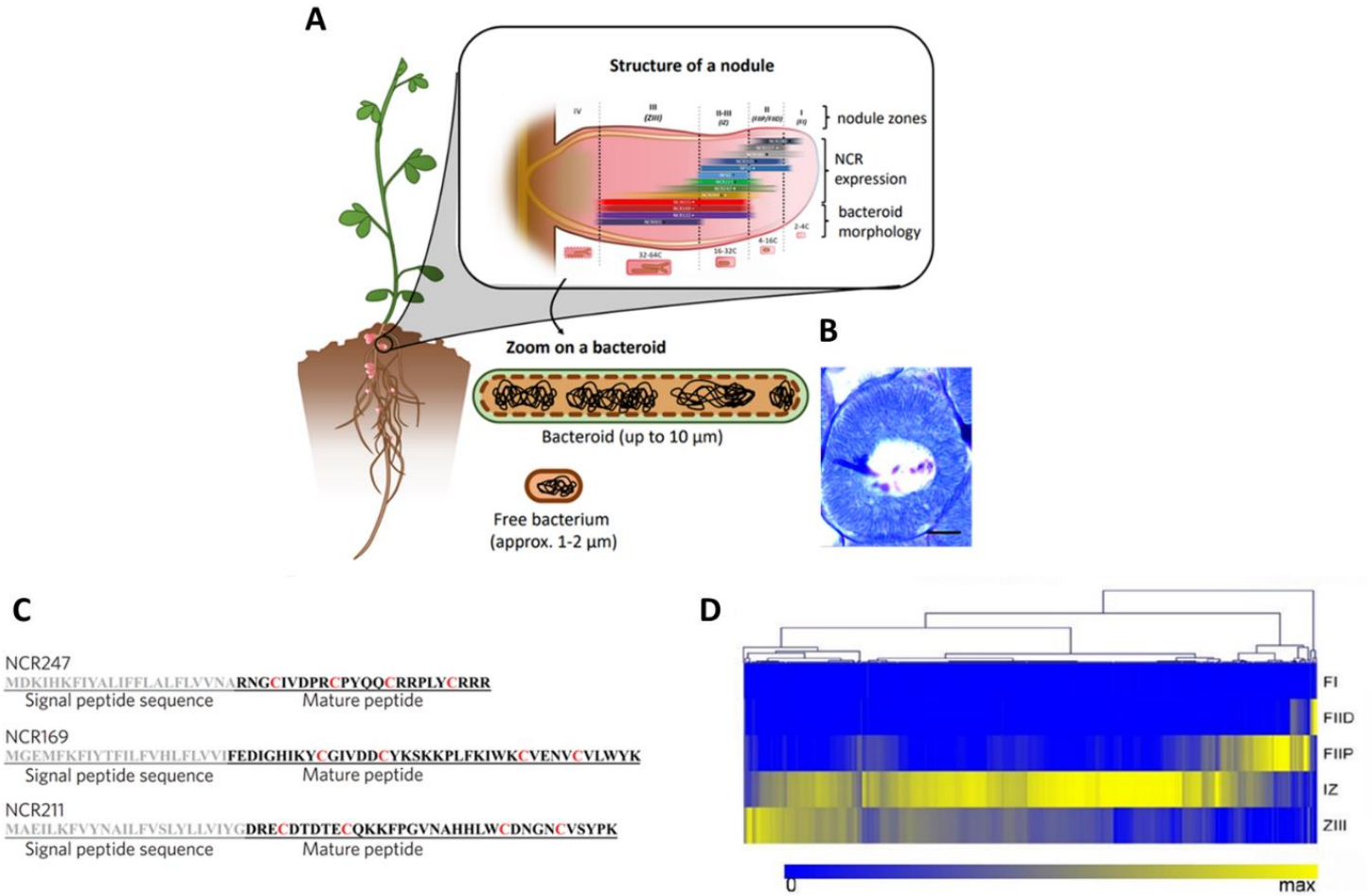


Figure 21: The symbiosis between *Sinorhizobium meliloti* and *Medicago sativa* leads to a bacterial differentiaction governed by the NCR peptides produced by the plant.

A) The symbiosis between the alphaproteobacterium *Sinorhizobium meliloti* and the legume *Medicago sativa* (on the left) produces a new organ called nodule. On the top panel, a zoom on the structure of a nodule and, indicated with colored bars, the expression of various NCR (Nodule-specific Cysteine-Rich) peptides are shown. Once in the infection zone, the bacterium begins a dramatic differentiation process under the influence of the NCR peptides leading to a bacteroid cell. A symbiosome (a zoom is represented in the bottom central part) is composed by a layer of plant membrane containing a bacteroid cell, characterized by (a) multiple copies of DNA, (b) a cell enlargement (ten times bigger than the free-living cell also showed here),(c) an inability to divide and (d) a higher permeability, as shown by a dotted envelope. B) Image of a symbiotic plant cell (vegetal cell wall in blue) full of bacteroids (blue); plant nucleus is white (Peter Mergaert, unpublished). Black bar corresponds to 10µm. C) Protein sequence of NCR247, NCR169 and NCR211. The signal peptide sequences are labelled in grey, mature sequences in black, and the cysteine residues in red (Pan and Wang, 2017). D) Expression profiles of *NCR* genes in the different zones (Guefrachi *et al*., 2014).

All tested species of the IRLC produce NCR peptides in their nodules. On the other hand, the *Aeschynomene* legumes of the Dalbergoid clade produce in their nodules NCRs of an unrelated family with distinct sequences and cysteine patterns (Czernic *et al.*, 2015; Gully *et al.*, 2018; Quilbé *et al.*, 2020).

Several arguments have confirmed the initial phylogenomic correlation between the production of NCRs in the symbiotic nodule cells and the terminal differentiation of bacteroids (Figure 21). A recent analysis for example showed that the degree of differentiation of bacteroids in species of the IRLC correlates with the number of NCRs expressed in nodules and also with the type of peptides they produce. The higher the diversity of peptides and the more cationic the NCR peptides are, the stronger the morphological change of the bacteroids (Montiel *et al.*, 2017).

NCR peptides are addressed to the bacteroids indicating that the endosymbionts are their target. Indeed, the localization of many individual peptides in the bacteroids or symbiosomes have been demonstrated by immunolocalization, by expressing NCR-fluorescent protein fusions as well as by cell fractionations of nodule extracts and purifications of bacteroids followed by Western blot analysis or proteomics (Van de Velde *et al.*, 2010; Haag *et al.*, 2011; Durgo *et al.*, 2015; Czernic *et al.*, 2015; Horváth *et al.*, 2015; Kim *et al.*, 2015; Wang *et al.*, 2017; Yang *et al.*, 2017).

In vitro experiments have shown that NCRs induce features on *S. meliloti* that mimic the terminal bacteroids such as cell elongation and polyploidy *in vitro* (Van de Velde *et al.*, 2010; Haag *et al.*, 2011; Penterman *et al.*, 2014; Montiel *et al.*, 2017). Moreover, transferring *NCR* genes to *Lotus japonicus*, a legume that does not have them and that makes normally reversible bacteroids, leads to new bacteroid features similar to terminal bacteroids (Van de Velde *et al.*, 2010).

Complementary to these “gain-of-function” methods, also loss-of-function experiments are confirming the key role of NCR peptides in bacteroid differentiation. NCR peptides are secretory peptides, which depend on their signal peptide to be taken in charge by the secretory pathway for trafficking to their cellular destination. In the *M. truncatula* mutant of the secretory pathway *dnf1*, NCR transport to the bacteroids is blocked. Thus, NCRs are stuck in the endoplasmic reticulum in the infected nodule cells and this prevents terminal bacteroid differentiation (Van de Velde *et al.*, 2010; Wang *et al.*, 2010). Similarly, downregulation of the orthologous secretory pathway gene *DNF1* in *Aeschynomene* nodules by RNAi blocks

bacteroid differentiation (Czernic *et al.*, 2015). More recently, several mutants or allelic variations in particular *NCR* genes were identified in *M. truncatula* that affect the bacteroid differentiation and persistence (Horváth *et al.*, 2015; Kim *et al.*, 2015; Wang *et al.*, 2017; Yang *et al.*, 2017; Wang *et al.*, 2018a). These are very surprising findings in light of the high number of *NCR* genes in *M. truncatula*, which would intuitively lead us to suppose a very high level of redundancy, but they provide very strong support for the key role of the peptides in the bacteroid formation.

10- Rewiring the bacterial cell cycle during bacteroid differentiation

From the bacterial side, several factors were identified that are involved in the response of bacteroids to this assault of NCR peptides (Mergaert, 2018). They can be divided in three broad categories of functions. First, the NCR peptides interfere with CtrA and its regulon, FtsZ and several metabolic processes including protein synthesis, energy household and nitrogen fixation itself (Penterman *et al.*, 2014; Farkas *et al.*, 2014). Second, as mentioned above NCRs are similar to antimicrobial peptides and they indeed have antimicrobial activity. *S. meliloti* bacteroids use several mechanisms to protect themselves against this harmful activity of the NCRs such as BacA and YejABEF peptide transporters, the lipopolysaccharide outer membrane, and the stress response regulator RpoH1 (Haag *et al.*, 2011; Montiel *et al.*, 2017; Arnold *et al.*, 2018; Nicoud *et al.*, 2021). Finally, the polyploid state of the bacteroids implies that the terminal bacteroid differentiation is driven by a switch in the bacterial cell cycle whereby the regular cycle composed of sequential steps of a single genome replication followed by cell division is transformed into a process of repeated genome replications without cell divisions (Mergaert *et al.*, 2006). In the next sections, we first discuss the state of the art of our knowledge of the regular cell cycle control in *S. meliloti* and then we will analyze the available data that highlight how the NCR peptides can interfere with the cell cycle to promote bacteroid differentiation.

The polyploidy of the *S. meliloti* bacteroids is a deviation of the single round of genome replication per cell division rule, which is governed as discussed above by CtrA and other master regulators. Thus, the cell cycle switch underlying the bacteroid differentiation should perturb this cascade. Most tellingly, the cell cycle switch is clearly observable by analyzing inside nodule tissues the expression of an extended set of *S. meliloti* cell cycle regulators. The meristem of *M. truncatula* nodules continuously generates new cells, which become infected and in which bacteria differentiate. This differentiation takes place gradually along the

longitudinal axis of the nodule. The expression of plant and bacterial genes was analyzed by combining laser-capture microdissection of nodule tissues along this longitudinal axis with RNA-seq (Roux *et al.*, 2014). A uniform expression of the cell cycle genes in all tissues would be expected in case the cell cycle stays unaffected during bacteroid differentiation. However, this is not what is observed (Figure 22). Rather, the relative expression of genes greatly varies in the different tissues and thus according to the stage of differentiation the bacteria. This expression modulation is gene- and thus cell cycle function dependent. For example, the expression of *ctrA* as well as many CtrA-regulated genes or genes encoding CtrA phosphorylation regulators is very rapidly downregulated when differentiation starts. Accordingly, Western blot analysis confirmed the absence of the CtrA and FtsZ proteins from bacteroids extracted from *Medicago* nodules (Pini *et al*. 2013; Farkas *et al*. 2014). The DNA replication associated genes *dnaA*, *dnaN* and *hdaA* show the strongest relative expression in the nodule tissues where differentiation and thus genome amplification is taking place.

The cell cycle regulatory cascade is a robust machinery that allows the strict respect of the haploid state-replication-diploid state-division cycle characterizing wild type *S. meliloti* growth. However, genetic or pharmacological interference with this cascade can disrupt the regular cell cycle and induce bacteroid-like cells, which are strongly enlarged and branched and have a multiplied genome while at the same time further growth and cell divisions are blocked. For example, the depletion of *ctrA* has such an effect (Pini *et al.* 2015). Also depletion of the *ctrA*-transcriptional regulators *gcrA* and *ntrX* or overexpression of *ccrM* or the sRNA EcpR1 provoke the same defects (Wright *et al*., 1997; Robledo *et al.*, 2015; Xing *et al*., 2020). Furthermore, the overexpression of *divJ*, the depletion of *pleC* or the mutation of *podJ1*, which encode negative regulators of CtrA phosphorylation and thus activation, have a similar effect on bacterial cells (Fields *et al.*, 2012; Pini *et al.*, 2015). Similarly, the expression of a constitutively active form of CpdR1 that stimulates CtrA degradation induces similar phenotypes (Kobayashi *et al.*, 2009). Furthermore, mutation or overexpression of the septum-formation controlling genes *ftsZ* and *minCDE* or pharmacological inhibition of septum formation again provoke the same cell elongation and branching effect (Latch and Margolin, 1997; Cheng *et al.*, 2007). Finally, overexpression of the *dnaA* and *hdaA* genes, encoding the replication machinery, have also such a cellular effect, although in that case the balance between the three replicons, the chromosome, pSymA and pSymB, is not maintained in the amplified genomes (Sibley *et al.*, 2006; Frage *et al.*, 2016; diCenzo *et al.*, 2022). Taken together,

perturbing the CtrA pathway leads systematically to cellular changes that mimic partially or strongly the bacteroid state.

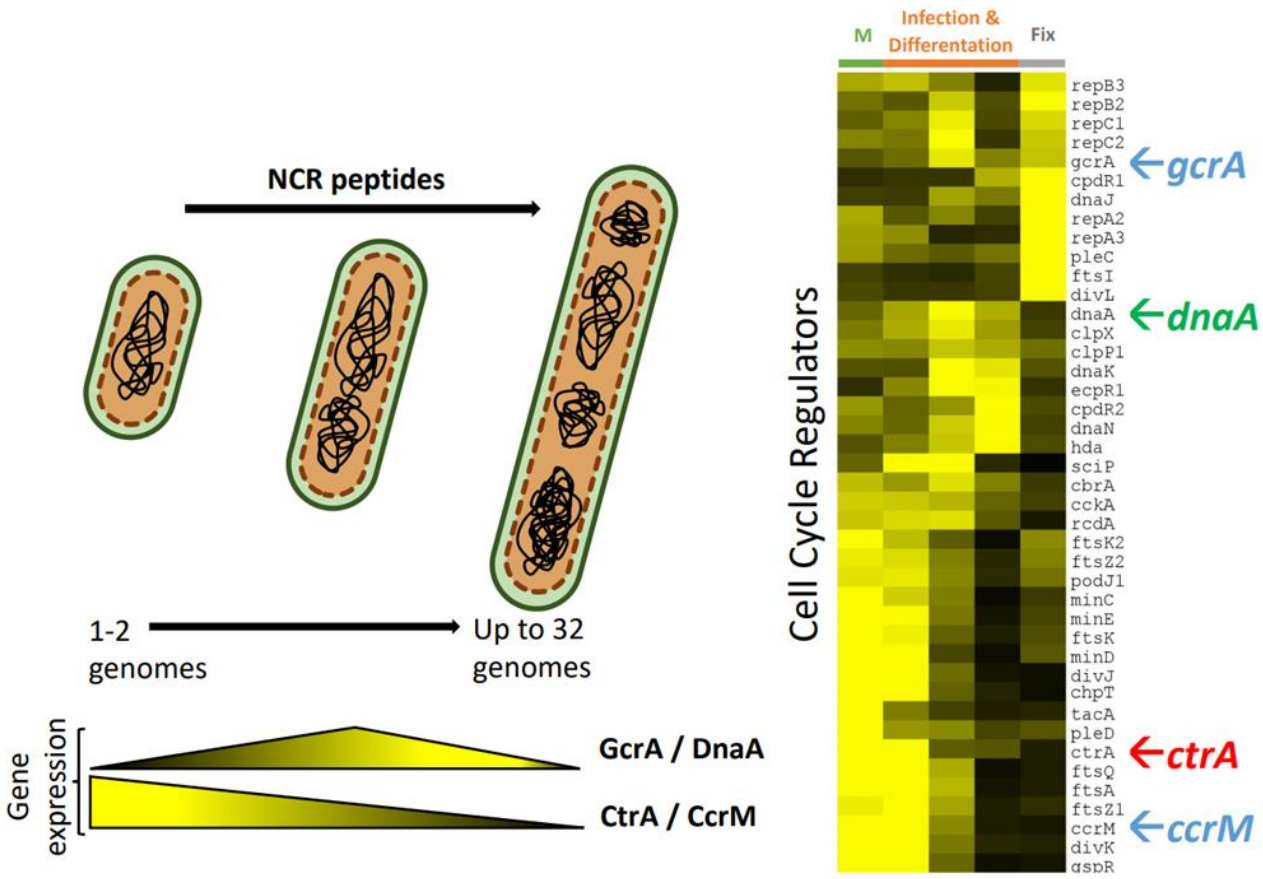


Figure 22: Gene expression pattern of cell cycle regulators during terminal bacteroid differentiation.

Under the impact of NCRs peptides, the bacterium differentiates into a bacteroid (upper left schematics). This differentiation consists of an endoreduplication and an absence of cell division resulting in a larger cell with up to 32 copies of the genome. Transcriptomic data of the cell cycle regulators genes in different nodule zone (on the right), shows a variation of the expression of cell cycle genes compared to the free-living growth condition (Roux *et al.*, 2014). In particular, *dnaA/gcrA* and *ctrA/ccrM* patterns are indicated in the lower left part of the figure (Dendene *et al.*, 2022).

Thus, CtrA, because of its key position in the cell cycle regulation, is a likely target for the NCR peptides in bacteroid differentiation: its elimination would be compatible with the inhibition of cell division and the continued DNA replication (Figure 22). As suggested by the

above-cited genetic studies, CtrA could be directly targeted or it could be inactivated and eliminated via its transcriptional or post-translational regulators. In agreement with the key position of CtrA, mutants in the *cbrA*, *divJ* and *cpdR* genes, each encoding negative regulators of CtrA, make non-functional nodules without bacteroid differentiation (Gibson *et al.* 2006; Kobayashi *et al.* 2009; Pini *et al*. 2013).

Another strong argument in favor of CtrA being the ultimate target of the NCR peptides to trigger the bacteroid differentiation, is the demonstration that in NCR247-treated synchronized *S. meliloti* cells the expression of the CtrA-controlled genes is not properly activated during the progression of the cell cycle (Penterman *et al.*, 2014).

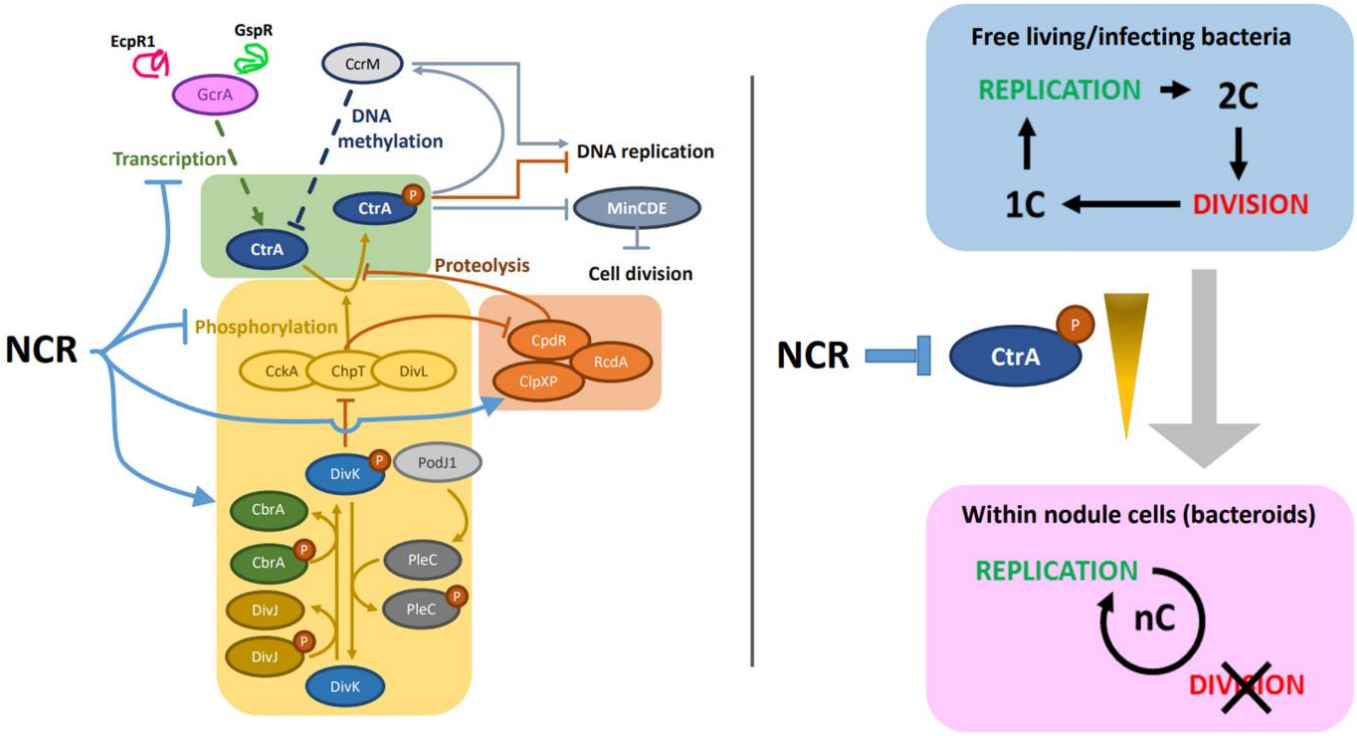


Figure 23: Schematics of the complex regulation network of the cell cycle in *Sinorhizobium meliloti* and the involvement of NCR peptides during the symbiosis process.

(A) CtrA is the master regulator of the cell cycle; its active form is phosphorylated and it directly inhibits the DNA replication and indirectly activates the cell division by inhibiting the expression of Min system, an inhibitor of the cell division. CtrA is strictly regulated to ensure a normal cell cycle progression. This regulation occurs at different levels, including a post translation level, by phosphorylation (yellow box) and proteolysis (orange box), as well as the transcription level through potentially GcrA and CcrM. The potential targets cell cycle of NCRs peptides are indicated with blue arrows. (Panel B) The free-living *S. meliloti* replicates its genome only once per cell cycle leading each time to two cell daughters (blue box). However, during the symbiotic process (pink box) the bacterium is targeted by NCRs, which lead to a drop of CtrA levels; as a result of this differentiation process the bacterium becomes a

bacteroid characterized by multiple copies of DNA (nC) and a cell division stop (Dendene *et al.,* 2022).

11- Benefits of terminal bacteroid differentiation

S. meliloti lives in the soil as a free-living saprophyte even without the presence of legumes (Carelli *et al.,* 2000). This suggests that the capability to establish a symbiosis is not an essential function of the species, as it is further revealed by the discovery of *S. meliloti* strains unable to induce and infect nodules. A recent study has highlighted that *S. meliloti* colonizes the plant as an endophyte, being recovered from leaves and other tissues from *Medicago* plants (Pini *et al.*, 2012). This observation suggests an interesting scenario about the evolutionary origin of bacteroid formation. The plant may have evolved a way to prevent uncontrolled infection of the endophyte, by blocking bacterial duplication and inducing a "terminal differentiation". Possibly, the NCR peptides that are now only active in nodules are derived from immune peptides that were originally employed to control endophytic bacteria including *S. meliloti* strains.

The multiple independent origins of terminal bacteroid differentiation in the legume family is a strong suggestion that the process provides benefits to the host plant (Oono *et al.*, 2011). The benefits should be indeed on the plant side because it is the host that imposes the process by the production of NCR peptides and the process limits very strongly the bacterial reproduction. However, it is possible that some advantages on the bacterial side are still to be discovered. Several studies have provided experimental confirmations that the terminal bacteroid differentiation improves the efficiency of the symbiosis by increasing the plant biomass production per investment in the symbiosis (Kobayashi *et al.*, 2009; Oono *et al.*, 2011). These analyses were comparative studies in which either a particular rhizobium strain was compared on two host plants (one inducing terminal bacteroid differentiation and the other not, or two hosts inducing different bacteroid types) (Sen and Weaver, 1981; Sen and Weaver, 1984; Oono *et al.*, 2011; Lamouche *et al.*, 2019a; Lamouche *et al.*, 2019b). Alternatively, *Medicago* hosts were nodulated with a panel of strains displaying contrasted bacteroid differentiation levels (different levels of genome amplification and cell enlargement in the bacteroids), correlating well with the efficiency of the interaction (Kazmierczak *et al.*, 2017). However, these comparisons although consistent with what we predicted, can be criticized as comparing apples with oranges because it is not possible to determine how much of the differences is due to bacteroid differentiation, and how much is due to other differences between the compared plant species/genotypes or bacterial species/strains. Moreover, they only show correlations,

which do not mean causality. To go beyond these correlations, an experimental system would be required that uses one particular host in interaction with one rhizobium strain and in which the bacteroid differentiation can be manipulated. Possibly, the *in planta* modification of the expression of cell cycle regulators, as discussed above in *in vitro* studies, can offer such opportunities.

Such an approach could provide a firm proof for the improvement of the symbiotic functioning of the bacteroids when terminally differentiated. However, this would still not explain why this type of bacteroids is performing better. This is at present an unresolved question but we can speculate about some of the consequences of the bacteroid differentiation that could affect the functioning of the bacteroids. The first possibility is that the advantage is linked to the cell size and that the cell enlargement makes bacteroids better nitrogen-fixing machines. Could larger bacteroids be more energy-efficient than small non-differentiated ones? This is not self-evident. Larger bacterial cells suggest a higher volume-to-surface ratio, but since respiratory energy production is a membrane process, an increased volume-to-surface ratio may be energetically not advantageous. On the other hand, if energy production would not be rate-limiting for nitrogen fixation in bacteroids, a larger volume could favor protein synthesis (for example for the massive production of the nitrogenase complex and its metal cofactors) by reducing its cost. Cell enlargement could also be viewed as a form of cell compartmentalization that physically separates the oxygen-requiring respiratory complexes from the oxygen-sensitive nitrogenase machinery. In a large cell, the nitrogenase could be located in the center of the cell, far away (on an atomic scale) from respiration on the cell membranes.

Additionally, large cells could dampen functional heterogeneity between bacteroids as recently demonstrated. Indeed, many bacterial genes show cell-to-cell fluctuations due to noise in gene expression, leading to phenotypic diversity between cells (Ackermann, 2015). Heterogeneity in a cell population can be advantageous to bacteria in certain circumstances, by providing adaptability to unpredictably changing environments. In the nodule however, it could be detrimental for the symbiosis and be associated with suboptimal performance of a subpopulation of bacteroids. In large cells such as terminally differentiated bacteroids, gene expression noise could be reduced by effectively averaging cell contents, as has been shown in polyploid division-blocked *Bacillus subtilis* mutants, resulting in a decreased functional heterogeneity between cells (Süel *et al.*, 2007).

Another possibility is that the polyploidy state may provide the improvement of bacteroid functioning. The respiration of bacteroids and the nitrogen fixation process itself by the nitrogenase are inevitable sources of reactive oxygen species (Matamoros *et al.*, 2003). Reactive oxygen species may induce deleterious mutations, which in the long term may affect the functioning of the bacteroids. Polyploid bacteroids could be less sensitive to DNA damage because they have multiple gene copies. Thus, the polyploid state of bacteroids could increase their longevity, which would imply a delayed senescence. The polyploid chromosomes in bacteroids could bring along also a benefit at another level. More condensed than the chromosomes in free-living rhizobia (Mergaert *et al.*, 2006), the polyploid chromosomes in bacteroids could function differently. Their compaction could have an epigenetic impact on for example gene expression. Indeed, bacteroids displayed a lower genome methylation level than free-living cells (diCenzo *et al.*, 2022).

On the other hand, the cell cycle switch with the ensuing polyploidy and cell enlargement could be only side effects of another important function of the NCRs on the bacteroids. The NCR peptides, as many other antimicrobial peptides, disturb the membrane integrity of bacteria (Van de Velde *et al.*, 2010; Mikuláss *et al.*, 2016) and this correlates with the known enhanced membrane permeability in terminally differentiated bacteroids (Mergaert *et al.*, 2006). The membrane permeabilization of the bacteroids could enhance the metabolic exchanges between the symbionts thereby favoring optimally the nitrogen-fixation metabolism of the bacteroids with the metabolism of the host cell (Mergaert *et al.*, 2017). Moreover, metabolite exchange can also be favored in the terminally differentiated bacteroids because they are individually enclosed in a symbiosome and have a much closer contact with the symbiosome membrane than undifferentiated bacteroids, which harbor multiple bacteria in a single symbiosome, reducing the direct contact of the bacteria with the symbiosome membrane. Furthermore, NCR peptides were reported to interact directly with several metabolic enzymes, including the ribosomes, chaperones, enzymes of the energy metabolism and the nitrogenase (Farkas *et al.*, 2014). Thus, the primary effect of the NCRs could be the manipulation of the metabolism of the endosymbiont in order to mold the bacterial metabolism for optimal nitrogen fixation (Kereszt *et al.*, 2011; Farkas *et al.*, 2014). For example, to maintain a redox balance during nitrogen fixation, bacteroids channel part of their carbon sources in lipid and polyhydroxybutyrate electron sinks (Terpolilli *et al.*, 2016). From the plant perspective, this accumulation of carbon by the bacteroids is a net loss of resources. It is striking that

undifferentiated bacteroids accumulate much larger amounts of these storage compounds than terminally differentiated bacteroids (Lodwig *et al.*, 2005).

A final hypothesis is related to the terminally differentiated state of the bacteroids. The terminal differentiation could limit the release of rhizobia from senescing nodules thereby moderating the impact of the symbiosis on the rhizosphere and endophyte microbiota. Moreover, the plant recovers during senescence the bacterial biomass from terminally differentiated bacteroids, which are entirely digested during nodule senescence (Van de Velde *et al.*, 2006) whereas undifferentiated bacteroids largely survive nodule senescence (Müller *et al.*, 2001).

12- Objectives of the thesis project

My thesis work was dedicated to the deciphering of "FcrX" function, a newly discovered cell cycle regulator in the alphaproteobacterium *Sinorhizobium meliloti.* The study of the cell cycle have been for a long time of a major interest. Indeed, the comprehension of this mechanism basis is essential to design therapeutics molecules to inhibit pathogens growth or in the contrary promote the growth of beneficial bacteria. Moreover, the knowledge acquired from these studies may be used to understand the cell cycle of eukaryotes. Indeed, the alphaproteobacterial cell cycle logic, reassembles the eukaryote's one. For many years, most alphaproteobacteria's cell cycle studies focused on the model *Caulobacter crescentus*. However, other models from the same class are becoming more investigated. *S. meliloti,* constitutes an interesting model especially due to its capacity to establish a symbiotic interaction with legumes plant. Thus, *S. meliloti* is a convenient model to better apprehend the mechanism of symbiotic interaction with legumes plant. Moreover, this understanding may help to develop new tools to promote the nitrogen-fixing symbiosis as an alternative to chemical fertilizers.

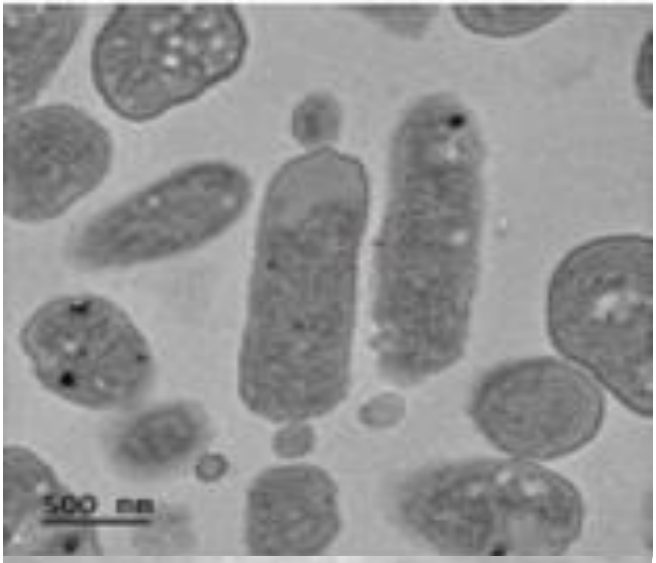
During this PhD thesis, two main biological questions were raised. First, we wanted to understand the involvement of FcrX in the cell cycle regulation in free-living conditions. Secondly, we wanted to understand its role during the symbiosis process with the host plant *Medicago sativa*. At the beginning of my PhD, little was known about the function of FcrX that had just been identifed. Previous experiments have shown a potential link between this latter and the proteins CtrA and FtsZ1 and 2, the master regulator of the cell cycle and the tubuline-like proteins, respectively. In order to unravel the link between FcrX and both CtrA and FtsZ 1

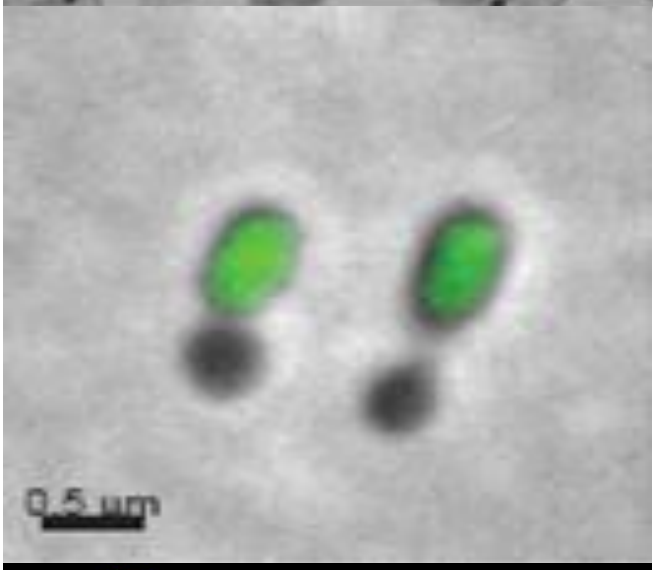
and 2, we used molecular biology approaches and phenotypic analysis. The conservation of FcrX among bacteria was also addressed by phylogenetic analysis together with experimental tests.

In order to, investigate the involvement of FcrX in the symbiosis process with *Medicago sativa*, plants were inoculated with strains expressing different levels of FcrX. The effect of FcrX deregulation on the symbiotic performance was evaluated by measurement of the plant dry weight, by determining the bacteroid DNA content and the cell size by flow cytometry, and the nodule colonization was analyzed by confocal microscopy.

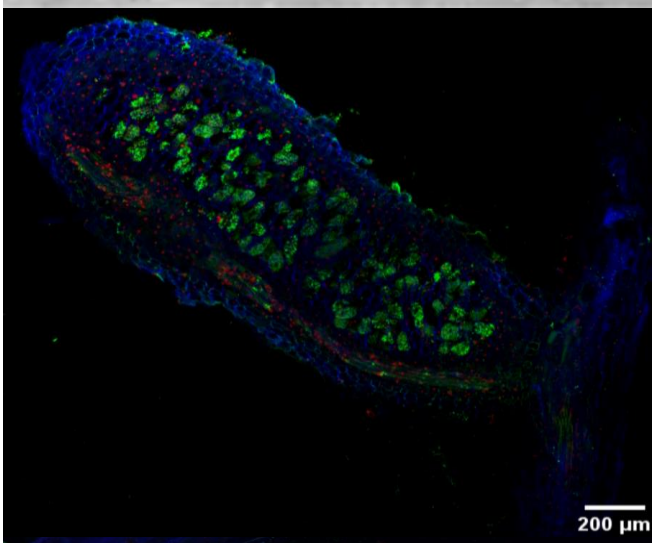
To go in depth in the deciphering of FcrX mode of action, we investigated whether additional factors may be involved in this function. To do so, we realized an interaction experiments and from this latters, the degradosome complex raised as a potential mechanism by which FcrX downregulates the accumulation of its targets. In order, to verify this hypothesis, stability of FcrX, in different mutant backgrounds was tested. Subcellular localization observations of the degradosome complex actors was also conducted.

Finally, a potential direct link between FcrX and the host was investigated, as well. Thus, the sensitivity of *S. meliloti* strains expressing different level of FcrX, was tested towards the NCR247 peptide. A plate reader monitored the results.





II- Results



a- Foreword

My thesis project has focused on to the study of a novel regulator FcrX in the alphaproteobacterium *Sinorhizobium meliloti*. The discovery of FcrX triggered our interest due to its obvious link to the master regulator CtrA. Indeed, FcrX possesses an interesting feature: its coding sequence is located nearby *ctrA* and both share the same intergenic sequence, which can suggest a common function. Moreover, a genetic screening realized on Δ*divJ* mutant revealed FcrX as a potential CtrA regulator so, a direct link to the cell cycle. For many decades, the cell cycle regulation of alphaproteobacteria constituted a subject of intensive studies. Indeed, the architecture of their cell cycle regulation is highly sophisticated and resembles the eukaryote's cell cycle, at least sharing the same logic (Biondi *et al.*, 2006). Thus, the finding of a novel regulator to be added in the already long list of regulators is extremely intriguing, particularly, in an organism that interacts with multicellular hosts like plants.

The alphaproteobacteria cell cycle regulation was mainly studied in the model *Caulobacter crescentus.* Thus, many data are available, which can enable us to apprehend this sophisticated circuit in other alphaproteobacterial models, and this with a greater ease. Indeed, many species from the *Alphaproteobacterial* class are of a major interest for the public health and the agriculture field, for example human and animal pathogens such as *Brucella abortus* and phytopathogens like *Agrobacterium vitis* and *A. tumefaciens*. However, many gaps remains in our understanding of their cell cycle, thus, it would be interesting to push our knowledge.

This work will be divided in two chapters; the first chapter, will describe a study conducted in order to understanding the basic function of FcrX in *Sinorhizobium meliloti* cell cycle regulation and it involvement during the symbiosis with the legume plant *Medicago sativa*. The second chapter, focused on the deciphering of the link between FcrX and the degradosome and the potential link between FcrX and the NCRs peptides. During my thesis project, we focused our work on the understanding of FcrX function and its positioning in the cell cycle regulation network. The first part of my research activity described in the first chapter, was submitted to the journal *Nature Communications* and is available in BioRxiv https://doi.org/10.1101/2023.03.13.532326. To conduct this study, a depletion strain of *fcrX* as well as several mutants were constructed by the Biondi laboratory and myself and characterized with a set of molecular and biochemical approaches that are details in the following submitted manuscript. We were able to decipher the function of *fcrX* and its involvement in the cell cycle

regulation of *S. meliloti* in free-living conditions. The flow cytometry and microscopy images acquisitions, together with their analysis were realized with the help of ImagerieGif facility. The phylogenetic tree was realized by our collaborator Matteo Brilli established at the University of Milan in Italy.

The ability of *S. meliloti* to establish a symbiotic relationship with legume plants makes it an even more interesting model. Taking into account the key position of FcrX in the cell cycle regulation, together with previous studies that highlighted the tight link between the cell cycle and the symbiosis process (Kobayashi *et al.*, 2009; Penterman *et al.*, 2014; Pini *et al.*, 2015), it was crucial to investigate FcrX function during symbiosis. To do so, I tested both the effect of FcrX depletion and overexpression during the symbiosis with its host plant *Medicago sativa*. These tests consisted in the measurement of the plant dry mass after 5 weeks post inoculation. A microscopy imaging of nodules section in order to appreciates the colonization efficiency of our mutants. Finally, I analyzed by flow cytometry the DNA content and the cell size of the same mutants purified from the nodules.

1- Sinorhizobium meliloti FcrX coordinates cell cycle and division during free-living growth and symbiosis

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b- Abstract

Sinorhizobium meliloti is a soil bacterium that establishes a symbiosis within root nodules of legumes (*Medicago sativa*, for example) where it fixes atmospheric nitrogen into ammonia and obtains in return carbon sources and other nutrients. In this symbiosis, *S. meliloti* undergoes a drastic cellular change leading to a terminal differentiated form (called bacteroid) characterized by genome endoreduplication, increase of cell size and high membrane permeability. The bacterial cell cycle (mis)regulation is at the heart of this differentiation process. In free-living cells, the master regulator CtrA ensures the progression of cell cycle by activating cell division (controlled by the tubulin-like protein FtsZ) and simultaneously inhibiting supernumerary DNA replication, while on the other hand the downregulation of CtrA and FtsZ is essential for bacteroid differentiation during symbiosis, preventing endosymbiont division and permitting genome endoreduplication. Little is known in *S. meliloti* about regulators of CtrA and FtsZ, as well as the processes that control bacteroid development. Here, we combine cell biology, biochemistry and bacterial genetics approaches to understand the function(s) of FcrX, a new factor that controls both CtrA and FtsZ, in free-living growth and in symbiosis. Depletion of the essential gene *fcrX* led to abnormally high levels of FtsZ and CtrA and minicell formation. Using multiple complementary techniques, we showed that FcrX is able to interact physically with FtsZ and CtrA. Moreover, its transcription is controlled by CtrA itself and displays an oscillatory pattern in the cell cycle. We further showed that, despite a weak homology with FliJ-like proteins, only FcrX proteins from closely-related species are able to complement *S. meliloti fcrX* function. Finally, deregulation of FcrX showed abnormal symbiotic behaviors in plants suggesting a putative role of this factor during bacteroid differentiation. In conclusion, FcrX is the first known cell cycle regulator that acts directly on both, CtrA and FtsZ, thereby controlling cell cycle, division and symbiotic differentiation.

c- Introduction

Sinorhizobium meliloti belongs to the class of *Alphaproteobacteria* and is known for its dual lifestyle: as a free-living bacterium in the soil and as a symbiotic endophyte within legumes of the genera *Medicago, Melilotus* and *Trigonella* (Alunni and Gourion, 2016). Free-living *S. meliloti* cells thrive in the soil and divide asymmetrically to produce two physiologically and morphologically different cell types (Figure 1A): a smaller cell, unable to replicate its DNA and a larger cell able to replicate its DNA once per cell cycle. The small cell first has to differentiate into the large cell type, upon suitable nutrient conditions, before it can undergo genome replication (Hallez *et al.*, 2004).

To ensure a normal progression of the cell cycle, it has to be tightly regulated. In alphaproteobacteria the response regulator CtrA plays a central role in this process by binding to the DNA, mostly activating or inhibiting transcription of more than a hundred target genes (Laub *et al.*, 2002; Brilli *et al.*, 2010). The function of CtrA has been mainly studied in *Caulobacter crescentus*, an aquatic bacterium that divides asymmetrically, as *S. meliloti*, giving two morphologically and physiologically different daughter cells (Hughes *et al.*, 2012); a stalked cell, able to replicate immediately after division, and a non-replicative motile cell that is blocked at the G1 phase. CtrA is under a strict control, as its levels oscillate during the cell cycle, reaching a maximum in two moments: i. at the G1 phase (motile cell), when it inhibits DNA replication by binding to specific sites that prevent origin binding of DnaA, the initiation factor of DNA replication, and ii. at the end of S-phase/G2, when CtrA also activates the cell division process (Quon *et al.*, 1998). To permit this pattern, regulation mechanisms at the transcriptional and post translational (by phosphorylation and proteolysis) levels are involved (Ryan *et al.*, 2004; Biondi *et al.*, 2006). An homolog of CtrA is present in *S. meliloti*, where it has a similar function (Barnett *et al.*, 2001; Pini *et al.*, 2015). In *S. meliloti*, CtrA inhibits indirectly the DNA replication by a yet-unknown process and activates the cell division by repressing the transcription of the *minCDE* system (Pini *et al.*, 2015), which ultimately inhibits the polymerization of the tubulin-like FtsZ responsible of cell constriction at the septum (Rowlett and Margolin, 2013). The FtsZ protein is composed by a N terminal core region, containing a GTPase domain, involved in the polymerization activity, and a C terminus responsible for the interaction to other actors (Sogues *et al.*, 2020). In *S. meliloti*, FtsZ is present in two copies, FtsZ1 and FtsZ2, however only FtsZ1 is essential for the Z ring formation, while the second copy lacks the C-terminal domain and its deletion is not lethal (Margolin, 2005). As

many cell cycle actors, FtsZ1 is expressed at the predivisional phase of the cell cycle (De Nisco *et al.*, 2014).

In symbiosis with *Medicago* plants, *S. meliloti* colonizes special root organs, called nodules. There, it fixes atmospheric nitrogen into ammonium that is assimilated by the plant, while it receives in return dicarboxylic acids and other nutrients (Long, 1989). In nodules, after a stage of multiplication, *S. meliloti* undergoes a drastic cellular change into a terminally differentiated form called bacteroid. This process takes place intracellularly, inside the nodule plant cells, where differentiated bacteroids are characterized by genome endoreduplication, cell enlargement and high membrane permeability (Mergaert *et al.*, 2006). Previous studies have shown an implication of the bacterial cell cycle regulation in this differentiation process (Pini *et al.*, 2013-2015; Kobayashi *et al.*, 2009). Indeed, CtrA and FtsZ are absent in bacteroids (Pini *et al.*, 2013; Farkas *et al.*, 2014) and mutants that overexpress CtrA are characterized by a symbiotic defect (Pini *et al.*, 2013). Interestingly, the depletion of *ctrA* in *S. meliloti*, similarly to *C. crescentus*, leads to a cell elongation/enlargement and endoreduplication phenotype that is strikingly similar to bacteroids formed in nodules. The mechanism leading to the downregulation of CtrA and FtsZ in bacteroids is not known yet but studies showed the involvement of plant-produced Nodule-specific Cysteine-Rich (NCR) peptides (Van de Velde *et al.*, 2010; Penterman *et al.*, 2014) Legumes such as *Medicago truncatula* produce a wide spectrum of NCR peptides (about 600) that are implicated in the disruption of several cellular processes including the cell cycle, thus resulting in the terminal differentiation of *S. meliloti* (Mergaert *et al.*, 2006; Van de Velde *et al.*, 2010; Alunni and Gourion, 2016). Indeed, the treatment of a wild type strain of *S. meliloti* with the NCR247 showed a down regulation of CtrA and its regulon. Farkas and colleagues also highlighted a physical interaction between this NCR and FtsZ (Farkas *et al.*, 2014). Overall these data strongly suggest that the regulation of bacterial cell cycle and cell division are playing a major role in the symbiosis process (Xue and Biondi, 2019).

Here we characterized the role of a new cell cycle regulator, named FcrX, elucidating its role with respect to CtrA and FtsZ1 and FtsZ2, its regulation by transcription, its conservation across the class *Alphaproteobacteria* and finally, its role during symbiosis.

d- Results

The *fcrX* gene is essential and controls cell cycle in *Sinorhizobium meliloti*

The *fcrX* gene (SMc00655, 351 bp ORF coding for a 116 aa predicted protein) is adjacent to *ctrA* in the *S. meliloti* chromosome in a head-to-head orientation, with a *ca.* 500 bp long intergenic sequence that controls the transcription of both genes. The *fcrX* gene appears essential in free-living conditions, based on Tn-seq results (Figure S1A). In order to study the function of *fcrX*, we constructed a depletion strain by deleting the chromosomal copy of *fcrX* and expressing an extra copy of *fcrX* on a plasmid under the control of an IPTG-inducible *lac* promoter (Khan *et al.*, 2008; Pini *et al.*, 2013). This conditional depletion strain of FcrX grew best in a medium supplemented with 100 µM IPTG (Figure S1B) and showed upon removal of IPTG dramatic growth defects (Figure 24B and Figure S1B), as well as a reduced capacity to form colonies (Figure 24C), indicating that *fcrX* is essential for the viability of *S. meliloti*. To gain more insights in FcrX function(s), we investigated the FcrX-depleted cells by microscopy and flow cytometry. Interestingly, the absence of FcrX induced the production of DNA-free small cells, referred to as minicells, as revealed by DNA staining by Syto9 and microscopy observation (Figure 24D). The accumulation of minicells in an FcrX-depleted suspension was confirmed by DNA/membrane double staining with DAPI/Potomac Gold dyes and flow cytometry analysis (Figure S2-S3B, 14.99% of total events with IPTG against 36.91% of total events without IPTG). Thus upon depletion of *fcrX*, an accumulation of small cells with no DNA was observed.

We monitored the depletion of FcrX by time-lapse microscopy in order to understand the development of minicells at the cellular level. Minicells tend to form only in daughter cells (small cell) while the mother (large) cell remains able to divide efficiently producing daughter cells, until two additional cycles and then completely stops dividing (Figure 24E). This minicell phenotype can be interpreted as the consequence of an imbalanced cell cycle, with an excess of division and a block of DNA replication, suggesting that FcrX may coordinate these two processes in *S. meliloti*. Consistently, the absence of FcrX led to increased levels of CtrA, FtsZ1 and FtsZ2 proteins, as shown by western blot analysis (Figure 25A), indicating that FcrX indeed negatively controls the accumulation of CtrA and FtsZ1/2. This result strengthens our hypothesis of the implication of FcrX in the regulation of cell cycle and cell division.

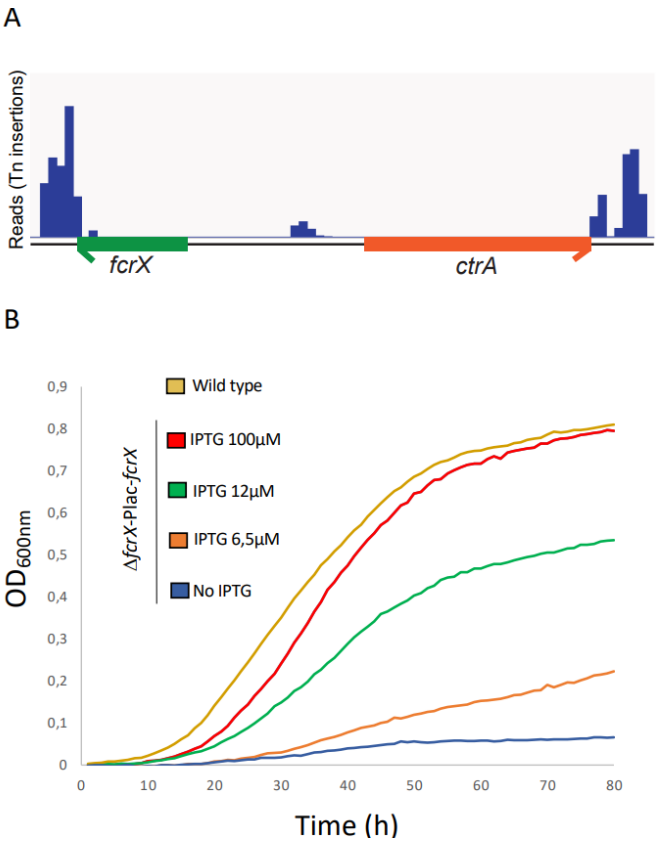


Figure S 1. Data supporting fcrX essentiality in *Sinorhizobium meliloti*.

(A) Schematics of the *fcrX-ctrA* locus with Tn-seq insertion frequency.

(B) Growth curves of *S.meliloti* Δ*fcrX*-P*lac-fcrX* strain cultivated with different IPTG concentrations. Wild type strain was used as positive contro

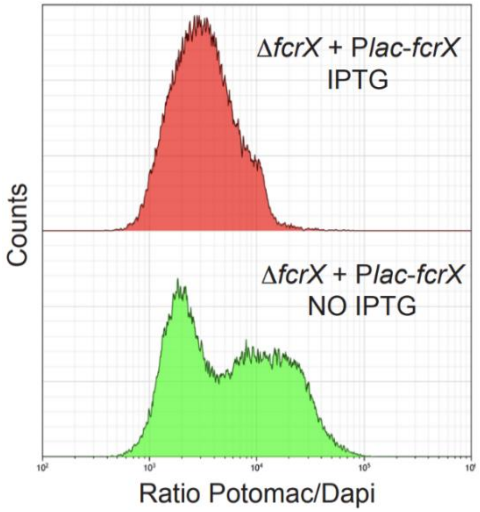


Figure S 2. Cells depleted of FcrX are empty of DNA.

Flow cytometry using Potomac (membrane) and DAPI (DNA) in Δ*fcrX*-P*lac-fcrX* with 100µM IPTG (red) and without IPTG (green).

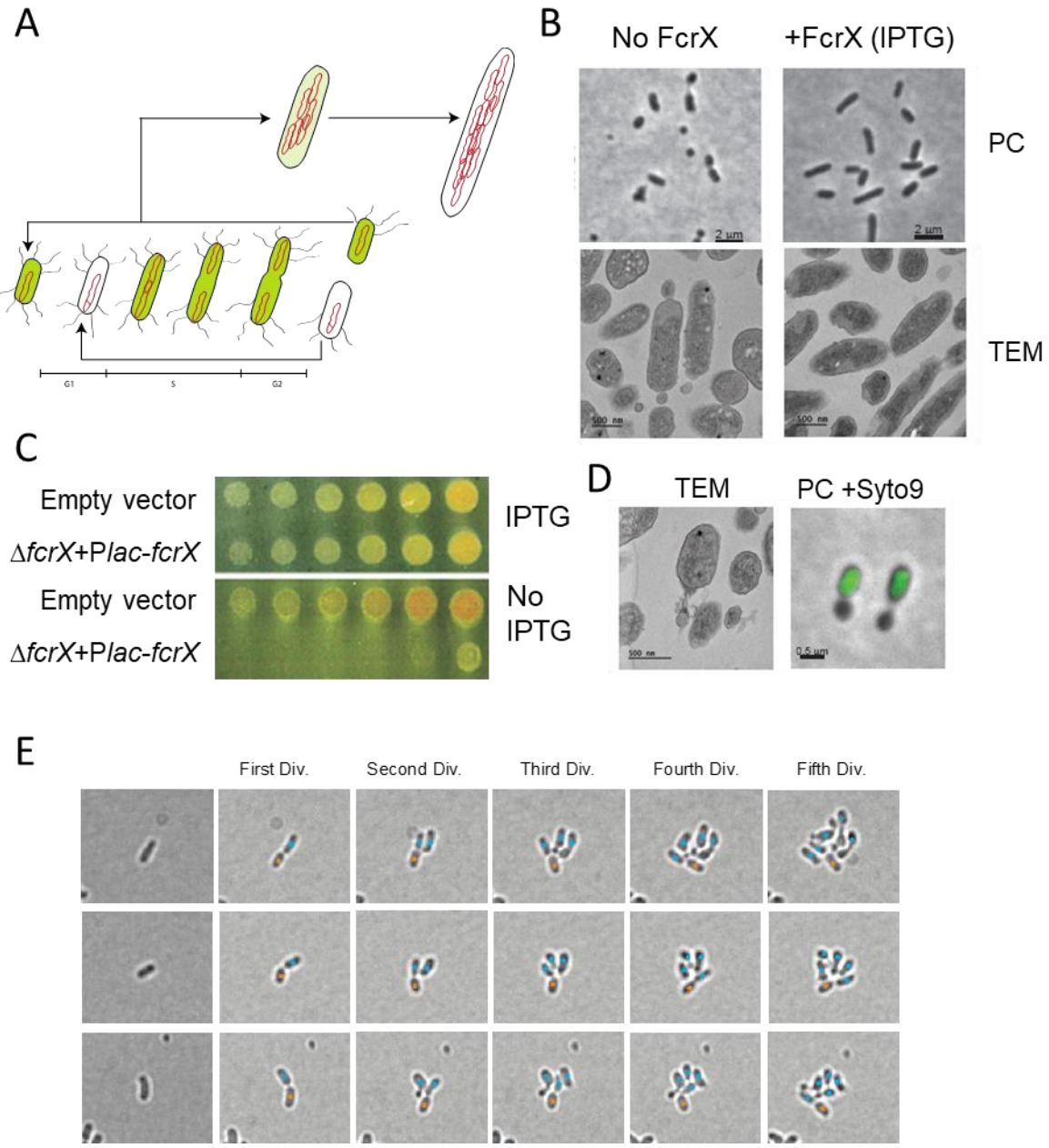


Figure 24: *fcrX* is an essential gene in *Sinorizobium meliloti.*

(A) Scheme representing the cell cycle progression of *S. meliloti* in free-living condition and in symbiosis with legume plants. The green color refers to CtrA concentration, which is low at the beginning of S-phase and after cell division in large cells. During bacteroid differentiation CtrA is removed in order to induce morphological changes.

(B) Phase contrast (PC) and transmission electron microscopy (TEM) of a depletion strain of *fcrX* in presence and absence of the inducer (IPTG). In particular, in no IPTG conditions, the depletion of *fcrX* is causing the formation of small cells (red asterisks).

(C) Viability test on *S. meliloti* containing empty plasmid and a depletion strain of *fcrX*. Cells of *fcrX* depletion strain or wild type strain carrying an empty plasmid were grown with

IPTG and then washed before plating. From left to right, non-diluted to 1/10^6 diluted cell suspensions were spotted on an agar plate with or without IPTG.

(D) Transmission electron microscopy (TEM) of a depletion strain of *fcrX* and overlay of phase contrast (PC) and epifluorescence microscopy of a depletion strain of *fcrX* labeled with Syto9.

(E) Time lapse microscopy on a depletion strain of *fcrX*. Orange dots represent the mother cells and the blue dots represent the daughter cells. Only daughter cells are able to produce abnormal mini cells. Scale bar corresponds to 2 µm.

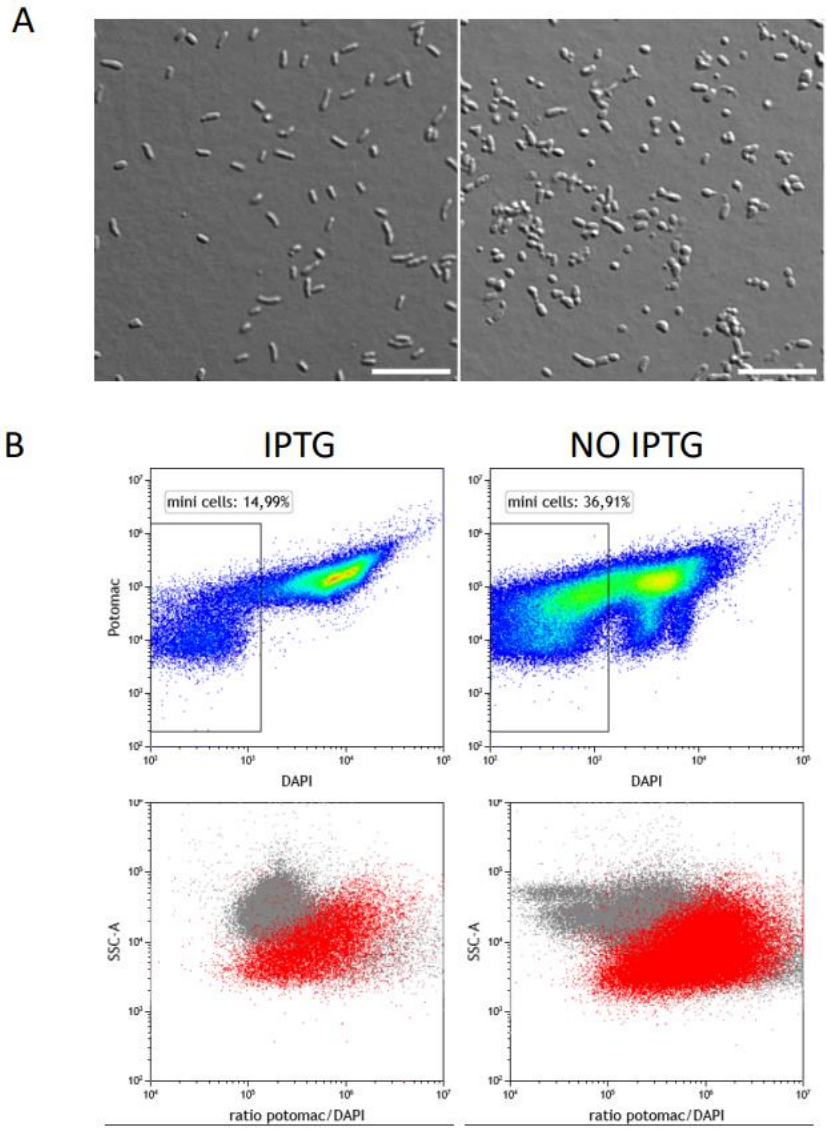


Figure S 3. Cells depleted of FcrX are minicells.

(A) Transmission contrast microscopy of Δ*fcrX*-P*lac-fcrX* with 100µM IPTG (left) and without IPTG (right). Bars correspond to 10µm.

(B) Flow cytometry analysis using Potomac (membrane) and DAPI (DNA) dyes in Δ*fcrX*-P*lac-fcrX* with 100µM IPTG (left) and without IPTG (right)**.**

FcrX interacts with CtrA and FtsZ1/2

The 3D structure prediction of FcrX performed with the Alphafold 2 algorithm showed that the FcrX protein may be composed of two alpha helices in a coiled coil configuration (Figure S4). The structure did not reveal the presence of a DNA binding domain, suggesting that its putative mode of action on CtrA and FtsZ1/2 may be by a direct interaction at the protein level. To verify this hypothesis, an affinity column experiment was realized using His6-FcrX loaded nickel columns in order to identify potential FcrX-interacting proteins. The presence of FtsZ1/2 and FcrX itself, together with other proteins listed in Table S1 was detected in the affinity column eluate by mass spectrometry (Figure S5). The presence of CtrA on the other hand was only detected by western blot (Figure 25B), as its low abundance makes it hardly detectable by mass spectrometry. In order to confirm this result, we carried out a bacterial two-hybrid (BACTH) experiment using the *Escherichia coli* carrier (Figure 25C) (Karimova *et al*., 1998). FcrX, CtrA and FtsZ1/2 proteins were fused to domains 18 and 25 of the adenylate cyclase from *Bordetella pertussis* in C- and N-terminal orientations and all possible combinations of prey and bait were introduced in the *E. coli* strain HB101. In this system, FcrX interacts with CtrA and with both copies of FtsZ (1 and 2). FtsZ2 lacks the C-terminal domain that is usually implicated in protein-protein interactions and recruitment of FtsZ partners (Xiao and Goley, 2016). Thus, the observed interaction between FcrX and FtsZ2 may be explained by either an unusual interaction between the N-terminal domain or by the presence of a functional full-length FtsZ homolog in *E. coli* ($FtsZ_{Ec}$), which could promote the formation of a FcrX-$FtsZ_{Ec}$-FtsZ2 ternary complex. We also tested the interaction of FcrX with itself to verify the possible dimerization of the protein, as observed with the affinity column. As shown in the (Figure 25C) a strong FcrX-FcrX interaction was observed, which is consistent with a putative oligomeric FcrX structure.

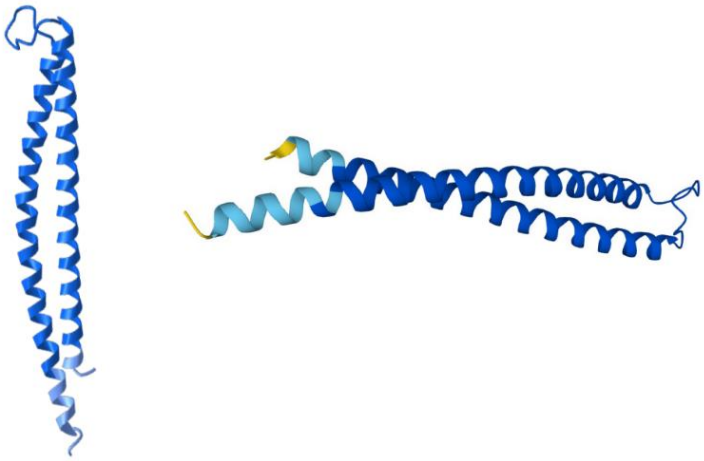


Figure S 4. Predicted structure of FcrX.

Structure of FcrX (from two different angles) was predicted using Alphafold2. Darker blue corresponds to higher prediction confidence.

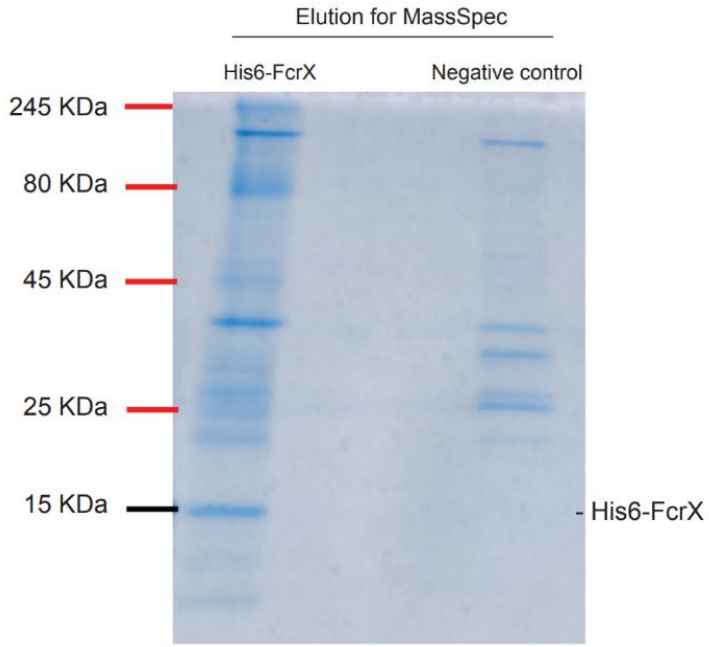


Figure S 5. Gel electrophoresis of His6-FcrX interacting proteins purified by affinity on a nickel column.

Affinity column experiments were performed as described in Materials and Methods. His6-FcrX (left) and negative control (right) were eluted from the column using 30% imidazole buffer.

Subcellular localization of FcrX and its interactors

To gain more insights about the function of FcrX we decided to investigate its subcellular localization and whether it colocalizes with its interactors, FtsZ being shown to have a mid-cell localization and CtrA potentially being polarly localized in *S. meliloti* as it has been shown previously in *C. crescentus* (Ma *et al.*, 1997; Wheeler *et al.*, 1998). We constructed a strain expressing a C-terminal translational fusion of FcrX with a yellow fluorescent protein (YFP) and further deleted the chromosomal copy of *fcrX* by transduction using a Δ*fcrX*::*tetR* M12 phage lysate. This approach was used in order to avoid all possible competitions of the wild-type FcrX with the tagged version and it confirmed that the fusion protein retained its function. FcrX localized at the septum, as observed by epifluorescence microscopy (with a less frequent localization at the cell pole, presumably after cell division) (Figure 25D). Image analysis in predivisional phase cells confirmed that FcrX is localized at the septum in the majority of these cells (Figure 25E). We wanted to correlate this result with the subcellular localization of the interactors over the cell cycle. Therefore, we constructed a C-terminal translational fusion of FtsZ1 with a cyan fluorescent protein (CFP), which despite the loss of its functionality retained its native localization (Ma *et al.*, 1997). Using a similar approach, we tagged FtsZ2 with CFP. CtrA localization investigation appeared more complex as N- or C-terminal tags strongly stabilized its levels, which is a lethal condition in *S. meliloti* (Pini *et al.*, 2015). Therefore, we added a sequence coding for the last 15 amino acids of CtrA that constitute the degradation motif, downstream of CFP to ensure the timely degradation of the fusion protein by proteolysis, then deleted the chromosomal copy of *ctrA* by transduction using a Δ*ctrA*::*tetR* M12 phage lysate. Tagged proteins were observed under the microscope. FtsZ1 and FtsZ2 showed as expected a mid-cell localization while CtrA showed a localization at the cell pole, consistently with FcrX localization (Figure S6). Together, these results indicate that FcrX may interact with both CtrA and FtsZ1/2 *in vivo* but at distinct subcellular localizations.

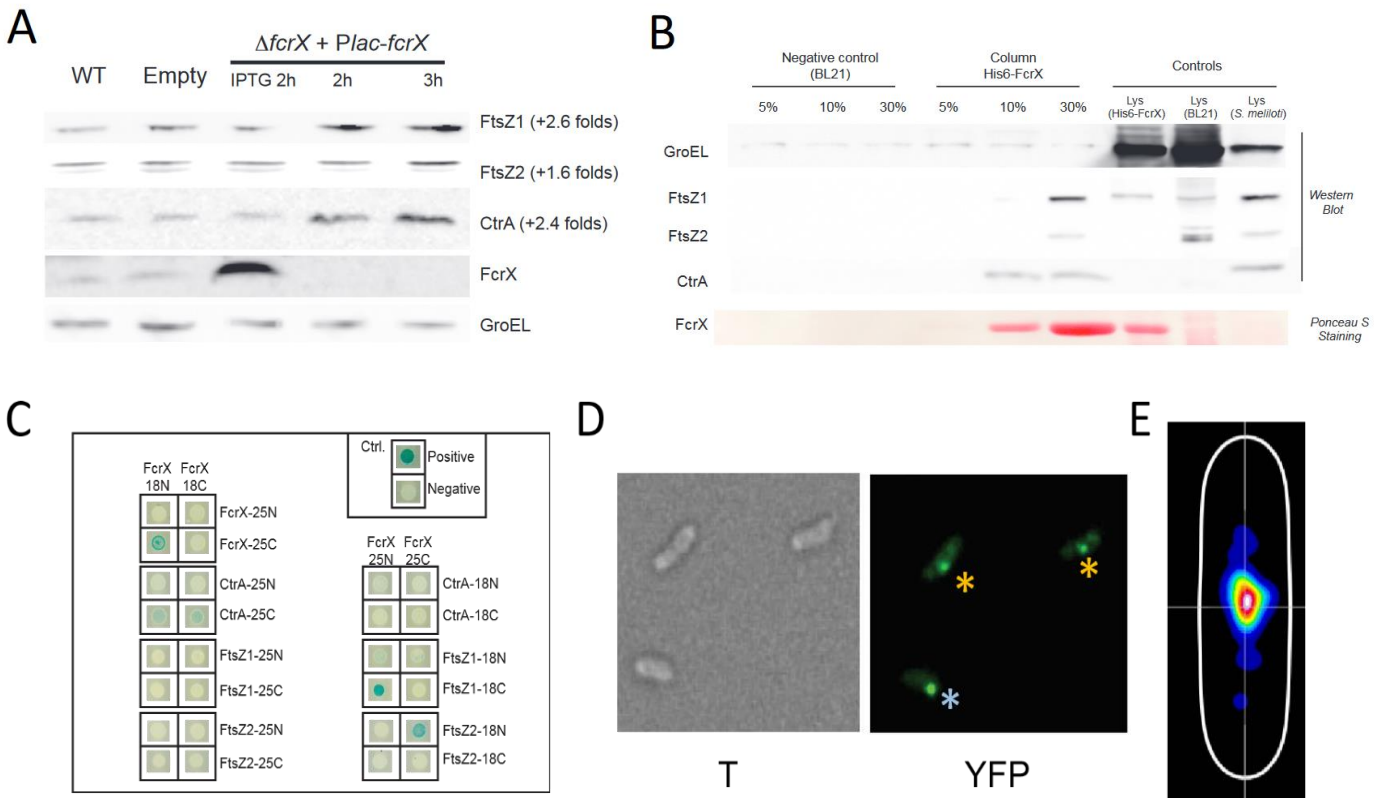


Figure 25: FcrX down regulates and interacts directly with the master regulator CtrA and the Z ring proteins (FtsZ1, FtsZ2).

(A) Western blot using Anti- FtsZ, CtrA, FcrX and GroEL on the *fcrX* depletion strain in comparison with wild type (WT) and a strain containing the empty vector used in the *fcrX* depletion strain (Empty). For the depletion strain of fcrX, a depleted culture was reincubated with 100µM IPTG for 2h (IPTG 2h) or without for 2h (2h) or 3h (3h).

(B) Affinity column western blot using CtrA, FcrX and FtsZ antibodies. Percentages represent imidazole concentration (see Materials and Methods for details). Membrane stained with Ponceau S shows His6-FcrX.

(C) Bacterial Two hybrid (BACTH) testing FcrX interaction with FtsZ1, FtsZ2, CtrA and itself. Upper right box shows negative and positive controls provided by the supplier. 25 and 18 are the two subunits of adenylate cyclase of the BACTH (see Materials and Methods).

(D) Functional FcrX-YFP C-terminal fusion observed by transmission (T) and epifluorescence microscopy (EM) in a *fcrX* deletion genetic background (see text for details). Bar corresponds to 1 µm. Cells showing mid-cell localization are marked with an orange asterisks, while the cell with polar localization is marked with a blue asterisk.

(E) Heatmap of YFP-FcrX subcellular localization in a synchronized cell population (predivisional phase, 150 minutes). Analysis performed on >300 cells (56 with foci).

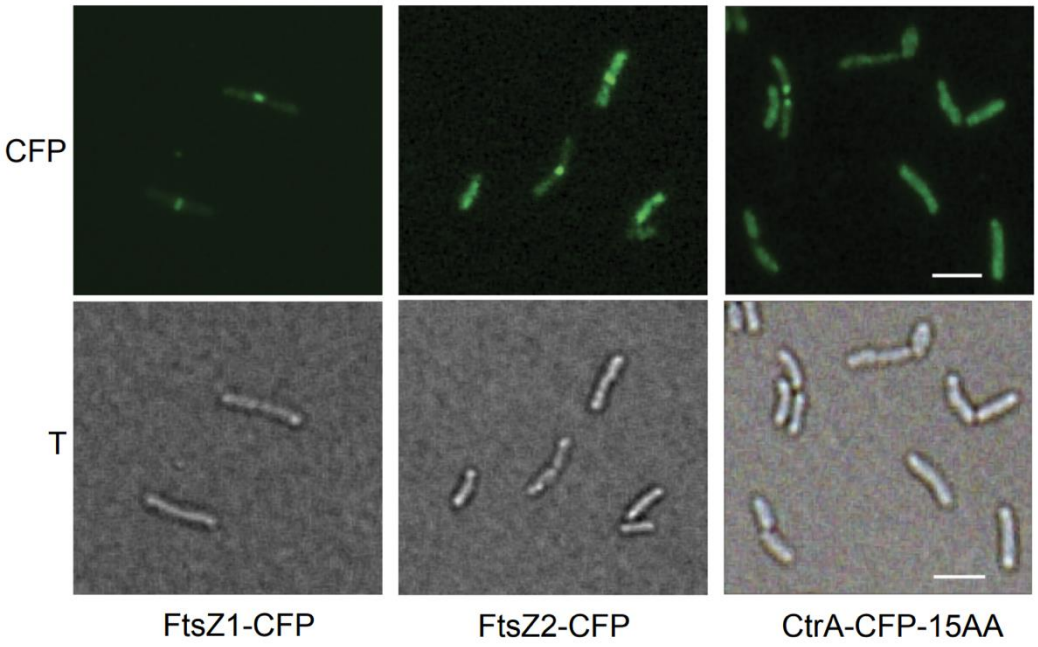


Figure S 6. Subcellular localization of FtsZ1, FtsZ2 and CtrA.

C-terminal translational fusions of FtsZ1, FtsZ2 and CtrA with CFP were constructed as described in the text. Scale bar corresponds to 2 µm. CFP and T refers to epifluorescence and transmission acquisition.

Transcription of *fcrX* is positively regulated by CtrA

In order to define and characterize the *fcrX* promoter, we made a promoter deletion analysis by introducing in *S. meliloti* wild-type cells an extra copy of *fcrX* with different putative promoter lengths located on a plasmid downstream of the IPTG-regulated *lacZ* promoter (Figure S7). Then, the obtained clones were transduced using a phage M12 lysate produced from the strain Δ*fcrX::tetR* + P*lac-fcrX* and selected in the presence or absence of IPTG. All clones should give viable colonies with IPTG while only the constructs containing a functional promoter of *fcrX* should support viability without IPTG (Figure S7). Constructs with P*fcrX-fcrX* fragments containing at least the region between -1/-287 were viable, while a shorter promoter fragment (-1/-224) was not active, suggesting that the promoter region of *fcrX* resides within the -287 region and that the -224/-287 contains a critical promoter element.

To confirm these results, we used the plasmid pOT1em (Meyer *et al.*, 2018), which contains genes encoding mCherry and EGFP in opposite directions. Several derivatives of the *fcrX* promoter, described in Figure S7, as well as the full intergenic region between *fcrX* and *ctrA*, were cloned between the ATGs of mCherry and EGFP. The full intergenic region between *fcrX*

and *ctrA* was able to express both mCherry (*fcrX*) and EGFP (*ctrA*) (Figure 26A). Analysis of the other constructs containing different versions of the upstream region of *fcrX* confirmed that only clones with at least -1/-287 expressed mCherry (*fcrX*) (data not shown). The *ctrA* gene is also controlled by the same intergenic region, suggesting that these two genes may share the same transcriptional regulation. The analysis of the region -224/-287 revealed several interesting characteristics. First, in this region the *ctrA*P1 promoter (Schlüter *et al.*, 2013) and the estimated *pfcrX* are overlapping with RNA-seq determined putative TSSs (transcriptional start site) separated only by 20/25 bp (Figure S7). Second, the promoter of *fcrX* contains a CtrA binding box within the 50 bp region upstream the putative TSS (Brilli *et al.*, 2010c; Pini *et al.*, 2015). These observations suggest that the activation of the *fcrX* promoter may depend on CtrA. In order to test this hypothesis, we mutated the CtrA-binding box by replacing the 5'-TTAA-3' half box with 5'-GCGC-3' in a pOT1em plasmid carrying the full intergenic region between *ctrA* and *fcrX* (Figure 26A). It was shown that this mutation prevents the fixation of CtrA on the box and therefore affects its transcriptional activity on the downstream gene (Figure 26A). Microscopy observation showed that the strain containing the mutated CtrA-binding box didn't express the mCherry fluorescence, implicating that CtrA may activate *fcrX* transcription by binding to its box. On the other hand, the mutated CtrA binding box did not change the EGFP expression noticeably, excluding the implication of this box in the regulation of *ctrA* transcription (Figure 26A).

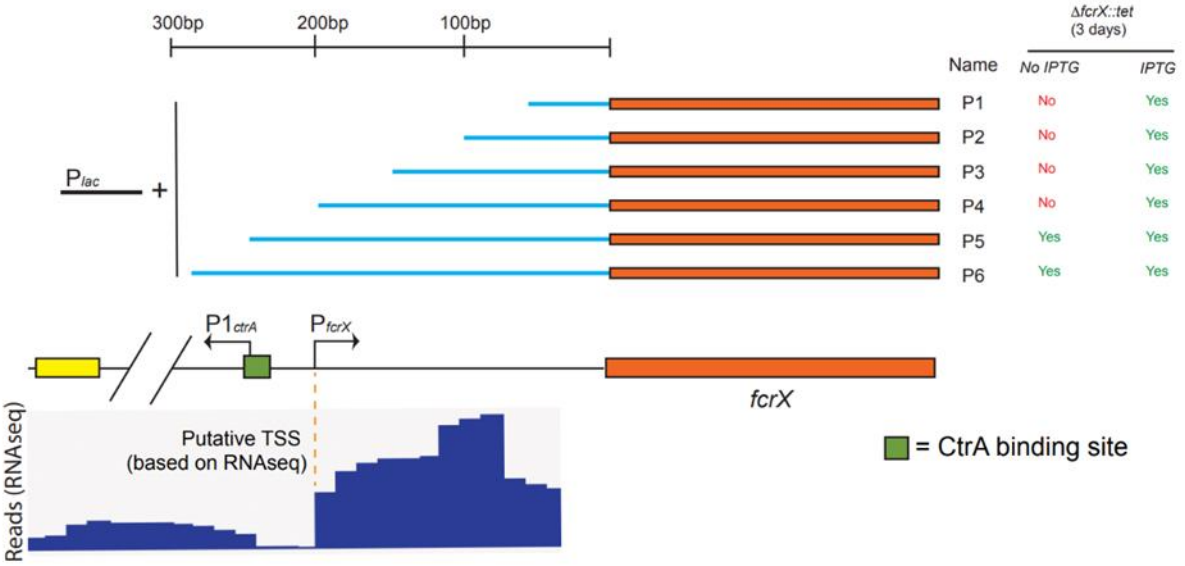


Figure S 7. Determination of *fcrX* promoter by deletion analysis.

Several complementation regions were constructed with different length of the putative *fcrX* promoter, adding also a P*lac* promoter (inducible by IPTG). A transduction lysate containing the *fcrX* gene locus replaced by the tetracycline resistance cassette was transduced in the different *S. meliloti* backgrounds containing the different *fcrX* constructs (results of the transduction on the right table with and without IPTG). See text for more details. At the bottom, an RNA-seq profile corresponding to the promoter region shows the consistency of RNA-seq profile with the genetics presented here.

To confirm the positive regulation of *fcrX* expression by CtrA, we tested the steady state levels of FcrX upon depletion of CtrA by western blot using antibodies directed against FcrX. In the absence of CtrA, we observed a significant decrease of FcrX (Figure 26B). Since CtrA is a DNA-binding protein implicated in transcriptional regulation, we also performed a qRT-PCR in the same conditions. Consistently, *fcrX* expression decreased upon CtrA depletion, confirming the previous observation (Figure S8). These results build up a regulatory model involving a negative feedback loop between CtrA and FcrX.

Cell cycle regulators are known to be dynamically regulated over the cell cycle, consistent with the oscillatory nature of this biological phenomenon. Because FcrX is closely linked to cell cycle regulation, we wanted to check whether FcrX was subject to oscillation. Therefore, a synchronization of a wild type culture of *S meliloti* was made as described before (Figure 26C) (De Nisco *et al.*, 2014). Samples were recovered every 30 minutes over a full cell cycle and the corresponding total RNA was used to perform a qRT-PCR analysis while cell

lysates were used for Western blot experiments using antibodies directed against FcrX. Both transcription and translation of FcrX increased at 90 min of the cell cycle, meaning that FcrX is not only a regulator of the cell cycle but it is also subject to cell cycle oscillation. These data are consistent with previous analyses on the of cell cycle regulated gene set (De Nisco *et al.*, 2014).

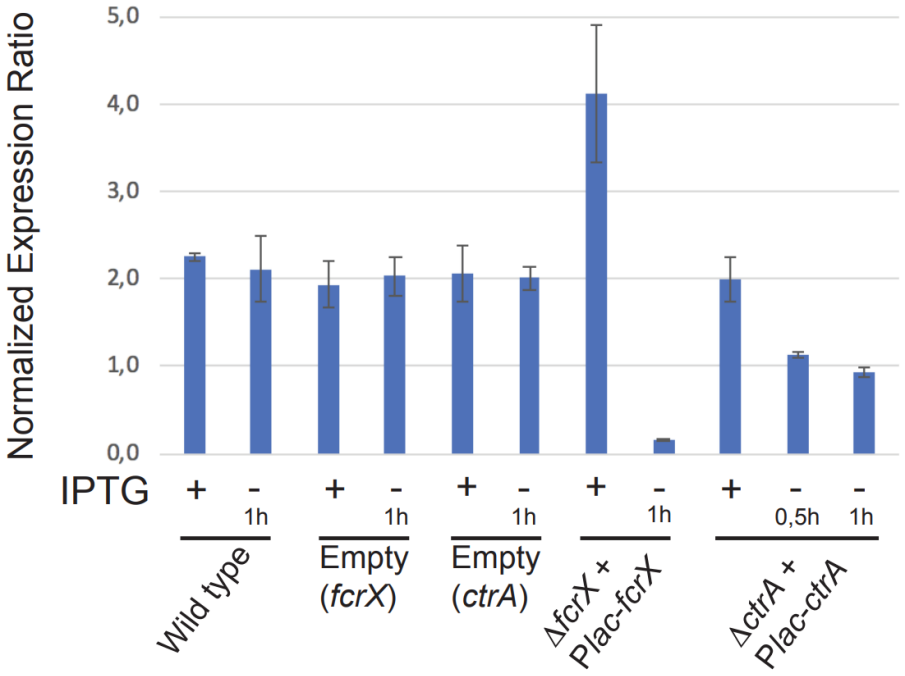


Figure S 8. Transcriptional levels of fcrX in different genetic backgrounds.

Expression transcriptional levels of *fcrX* normalized with respect to 16S rRNA were tested upon depletion of *fcrX* and *ctrA*. 100µM and 1mM IPTG was added to the depletion strain of *fcrX* and *ctrA*, respectively.

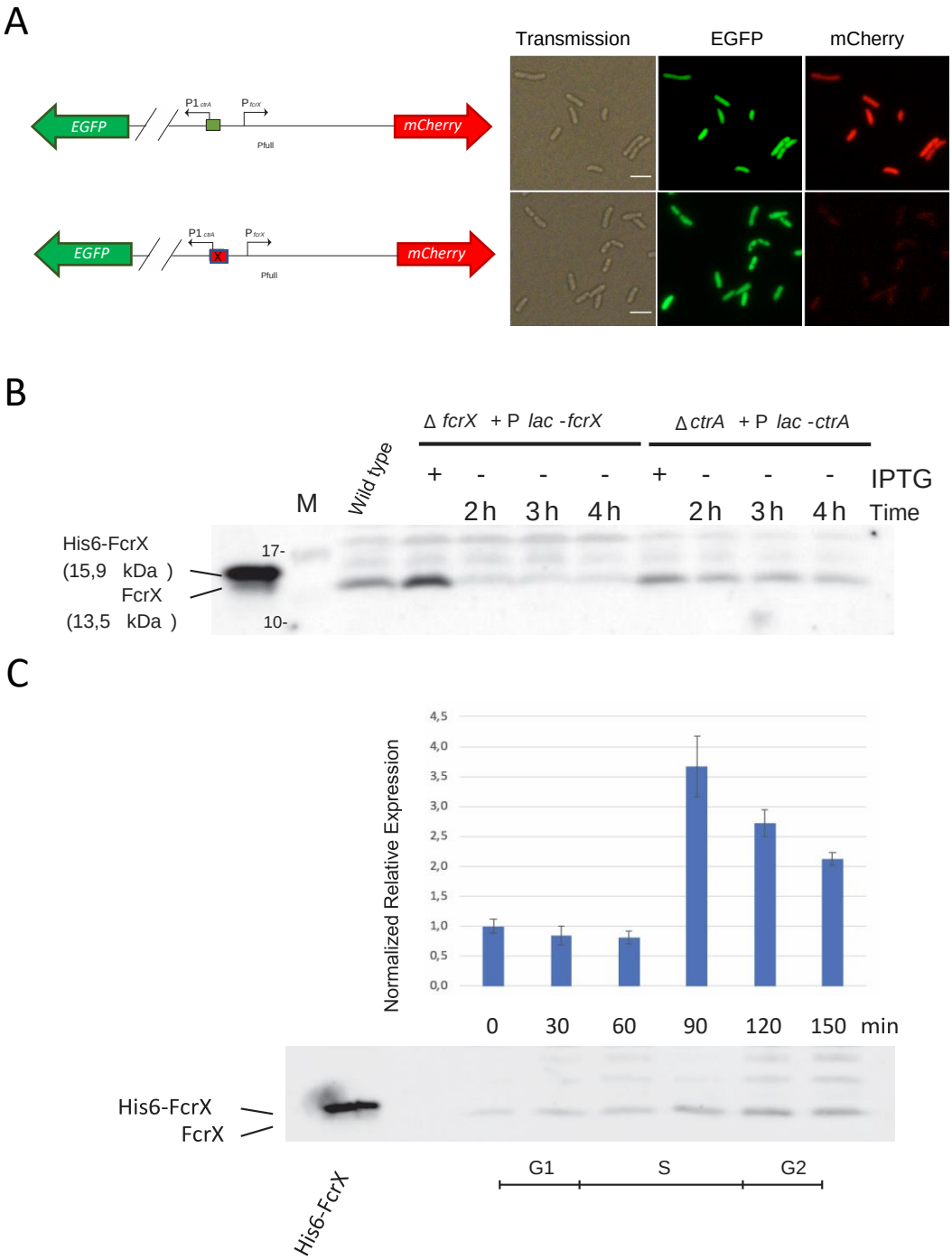


Figure 26: FcrX is cell cycle regulated and its transcription depends on CtrA.

(A) *S. meliloti* strain containing the intergenic region between *fcrX* and *ctrA* fused with mCherry and EGFP, respectively. Lower panels correspond to the same intergenic region mutated in the CtrA box (see text for details). This mutation does not affect the expression of *ctrA* but it completely abolishes the expression of *fcrX*.

(B) Western blot using anti-FcrX antibodies using FcrX depletion and CtrA depletion samples. First lane is purified His6-FcrX. M = Marker (sizes are reported).

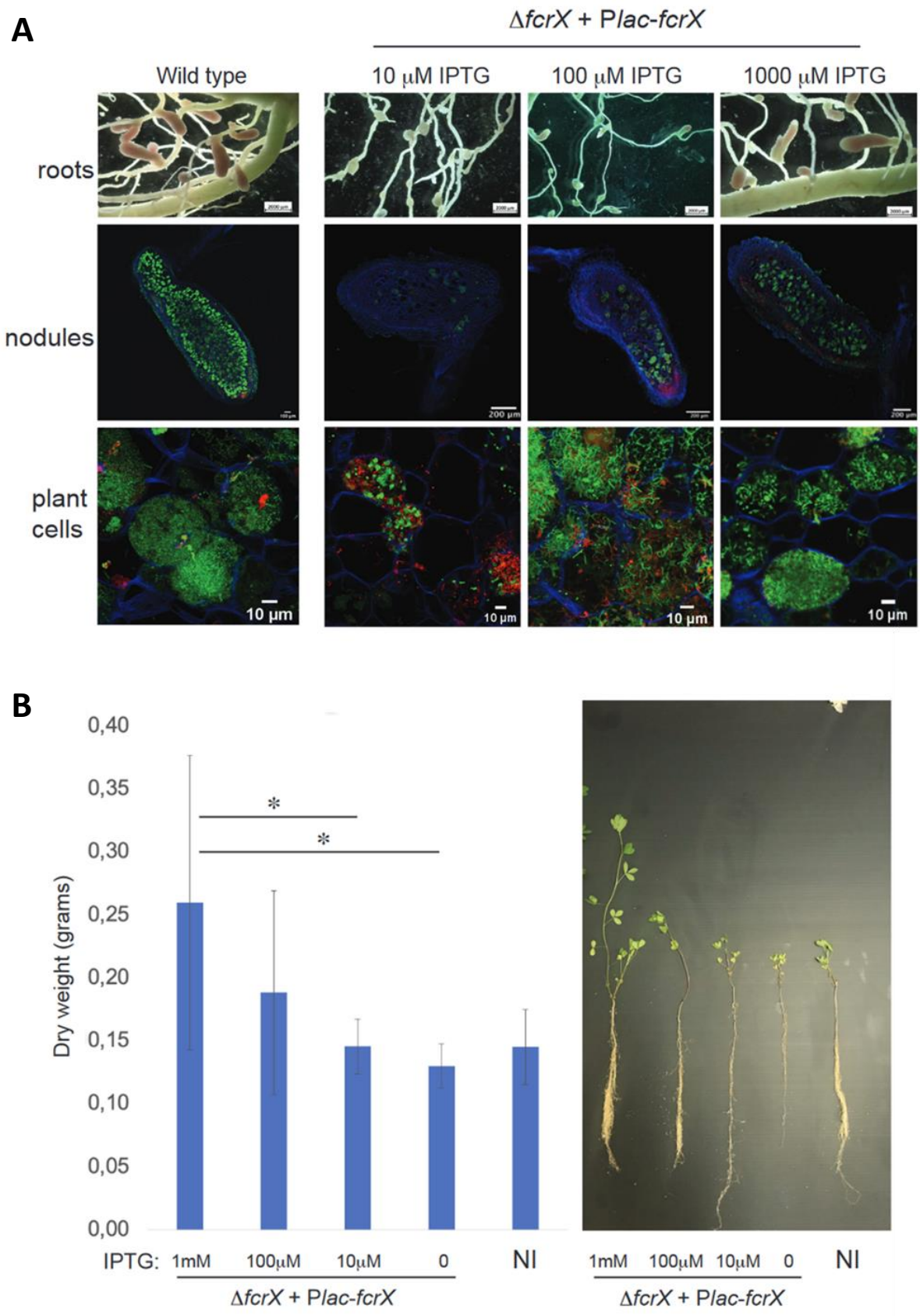
(C) qRT-PCR of *fcrX* and Western blot using anti-FcrX antibodies on a synchronized population of *S. meliloti.* Bottom part represents a timeline of cell cycle phases.

FcrX is essential for the establishment of the legume symbiosis

The terminal bacteroid differentiation that *S. meliloti* undergoes during its interaction with *Medicago* plants involves a remodeling of the cell cycle and its regulatory network, prompting us to test the involvement of FcrX in the symbiotic process. We tested the importance of FcrX by inoculating *M. sativa* plants with the FcrX depletion strain and watered the plants with a range of concentrations of IPTG in order to obtain different levels of FcrX expression. We also inoculated plants with the wild type strain of *S. meliloti* carrying an empty plasmid as a reference. At 28 days post inoculation (dpi) we checked nodule colonization using confocal microscopy (Figure 27A), measured the plant dry weight (Figure 27B) and assessed bacteroid differentiation by measuring the bacterial DNA content with flow cytometry (Figure S9). Plant weight decreased when FcrX expression was reduced with respect to the condition with the highest IPTG concentration (Figure 27B). The confocal microscopy images showed a clear nodule colonization defect, with much less plant cells containing bacteroids in the plants watered with 0µM, 10µM and 100µM IPTG compared to the plants watered with 1mM IPTG and the references, implying that FcrX is essential to the establishment of a fully functional symbiosis. However, the nodules from the condition watered with 1mM IPTG were still less colonized and the bacteroids did not show a similar extent of terminal differentiation as observed in the reference conditions, an observation that can be explained by the IPTG-regulated expression of FcrX, which may not mimic the natural cell cycle-regulated expression.

Considering our results showing that FcrX inhibits the accumulation of CtrA, FtsZ1 and FtsZ2 (Figure 25A), we wondered if an overexpression of FcrX may promote CtrA and FtsZ1/2 disappearance inside nodule cells and thereby boost the symbiotic process. To do so, we inoculated plants of *M. sativa* with a *S. meliloti* strain containing a second copy of *fcrX* expressed on a pSRK plasmid under the control of the P*lac* promoter, which has a weak transcriptional leakage in *S. meliloti*, even in the absence of IPTG (Khan *et al.*, 2008). The upregulation of FcrX resulted in a significant plant biomass gain at 28 dpi, as compared to the controls (Figure 27C). However, no increase in the bacteroid differentiation level was noticed, suggesting that the increase in symbiotic efficiency is likely related to the speed up of the differentiation process rather than to a larger extent of differentiation (data not shown). Finally,

we investigated whether fully differentiated bacteroids contained FcrX by using anti-FcrX antibodies. Unlike CtrA and FtsZ1/2 that are absent from mature bacteroids, FcrX remained detectable (Figure 27D), supporting a role of FcrX during the establishment of bacteroid differentiation and maintenance.


A
ΔfcrX + Plac-fcrX
Wild type
10 μM IPTG
100 μM IPTG
1000 μM IPTG
roots
nodules
plant cells
10 μm
B
Dry weight (grams)
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
*
*
IPTG: 1mM 100μM 10μM 0 NI
ΔfcrX + Plac-fcrX
1mM 100μM 10μM 0 NI
ΔfcrX + Plac-fcrX

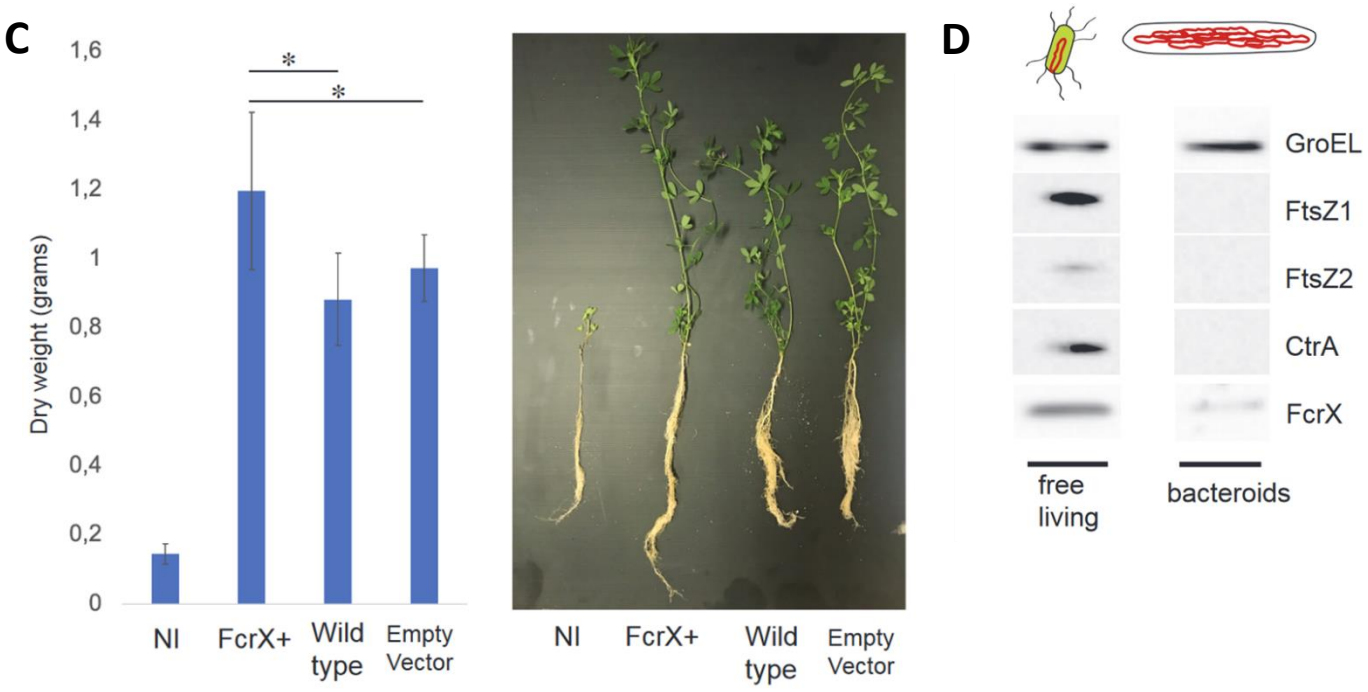


Figure 27: FcrX is important for symbiosis.

Nodules of 42 dpi plants infected by wild type (left row) and the depletion strain of *fcrX*, in different IPTG-watering conditions, were photographed (upper panels) and then sectioned and stained with Calcofluor, Syto9 and PI as explained in materials and methods (lower panels at high magnification levels).

(A) Dry biomass per plant (left panel) and aspect (right panel) of 42 dpi *M. sativa* infected by wild type and the depletion strain of *fcrX* at different IPTG concentrations. Asterisks correspond to significant differences (>20 plants per condition, $P<0.05$, Kruskall-Wallis test). NI = Non inoculated.

(B) Dry biomass per plant (left panel) and aspect (right panel) of 42 dpi *M. sativa* infected by wild type strain containing an empty vector (empty), wild type and a strain expressing an extra copy of *fcrX* (FcrX+). Asterisks correspond to significant differences (>20 plants per condition, $P<0.05$, Kruskall-Wallis test). NI = Non inoculated.

(C) Western blot using FcrX, FtsZ, CtrA and GroEL antibodies on free-living and bacteroid cells.

FcrX is a conserved factor in several species of the class *Alphaproteobacteria*

We further wanted to investigate the presence and functionality of FcrX in other bacteria. First, the *S. meliloti* FcrX protein sequence was used to search in the Microbes Online database (Dehal *et al.*, 2009) for similar proteins. Orthologs defined by Bidirectional Best Hit (BBH) of FcrX were found in several rhizobia (*Bradyrhizobium japonicum, Rhizobium leguminosarum*), the human pathogen *Brucella abortus* and the phytopathogen *Agrobacterium vitis*. Except for *A. vitis* for which no information is available, previous Tn-seq data on all those species revealed that the putative FcrXs were all essential in the organism of origin (Perry *et al.*, 2016; Sternon *et al.*, 2018; Baraquet *et al.*, 2021). Those orthologs were tested for their capacity to complement *fcrX* deletion in *S. meliloti*. We also tested a distant homolog of *fcrX* from *C. crescentus*, which codes for the flagellar protein FliJ, located in the same genomic context as *fcrX*. Interestingly, the *fcrX-ctrA* synteny is widely preserved among these bacteria suggesting a possible conservation of *fcrX* function (Figure 28A). In order to test our hypothesis, we constructed *S. meliloti* strains expressing a copy of these orthologs and we deleted by transduction the chromosomal copy of *fcrX*. Results of this experiment showed that the expression of the orthologs from *R. leguminosarum* and *B. abortus* were able to support *S. meliloti* growth in a *fcrX* deletion background, implying functional conservation of FcrX. A western blotting experiment was carried out confirming that the overexpression of these orthologs is able to down regulate the accumulation of FtsZ and CtrA (Figure S10). However, the orthologs from *B. japonicum*, *A. vitis* and the FliJ homolog from *C. crescentus* were not able to complement the *fcrX* deletion. This functional complementation analysis, although still limited in the number of tested species, revealed that from a functional point of view each FcrX has acquired specific functions independently from the phylogenetic distance from *S. meliloti*, as for example *A. vitis* (a species close to *S. meliloti)* is not able to complement while the more distant *B. abortus* does. In order to have a broader view of FcrX conservation, we searched for FcrX/FliJ orthologs/homologs in other alphaproteobacterial species (Figure 28B, Table S2). Significantly similar sequences to either the *S. meliloti* FcrX or the *C. crescentus* FliJ were only found in the *Caulobacterales* and *Rhizobiales*. However, the two queries retrieved sequences in complementary sets of species; for example, in *C. crescentus* FcrX retrieved no results (Table S2). This can be consequent to a functional diversification of the same ancestral gene in the *Caulobacterales* and *Rhizobiales*, which is however difficult to demonstrate because of the short length and variability of these sequences, even if a phylogenetic tree of all homologs found seems to suggest an orthology relationship as the topology is largely congruent with the

RecG tree (data not shown). In parallel we checked the distance between *fcrX/fliJ* and *ctrA* genes, discovering that the *ctrA* gene is in proximity to *fcrX* orthologs (or *fliJ* in *Caulobacterales*), and are often transcribed from the same intergenic region. Finally, we observed that orthologs able to complement the *S. meliloti fcrX* deletion belong to the phylogenetic group of *Brucella*-rhizobia (excluding bradyrhizobia). However, the functional complementation of *fcrX* is not a conserved feature of specific clades, as for example *A. vitis* does not complement. Finally, the functional diversification between FcrX and FliJ is also supported by the fact that, to the best of our investigations, FcrX has no role in the control of motility.

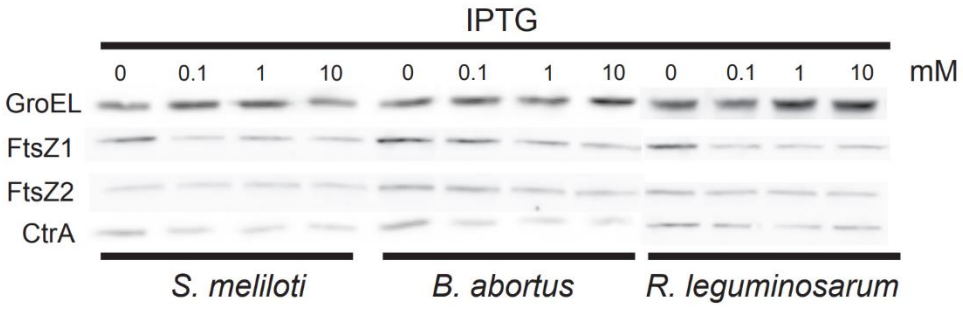


Figure S10: CtrA, FtsZ1 and FtsZ2 steady state levels in *S. meliloti fcrX* depletion strains complemented by *S. meliloti, B. abortus* and *R. leguminosarum fcrX*s. Western blots were performed as described in Materials and Methods

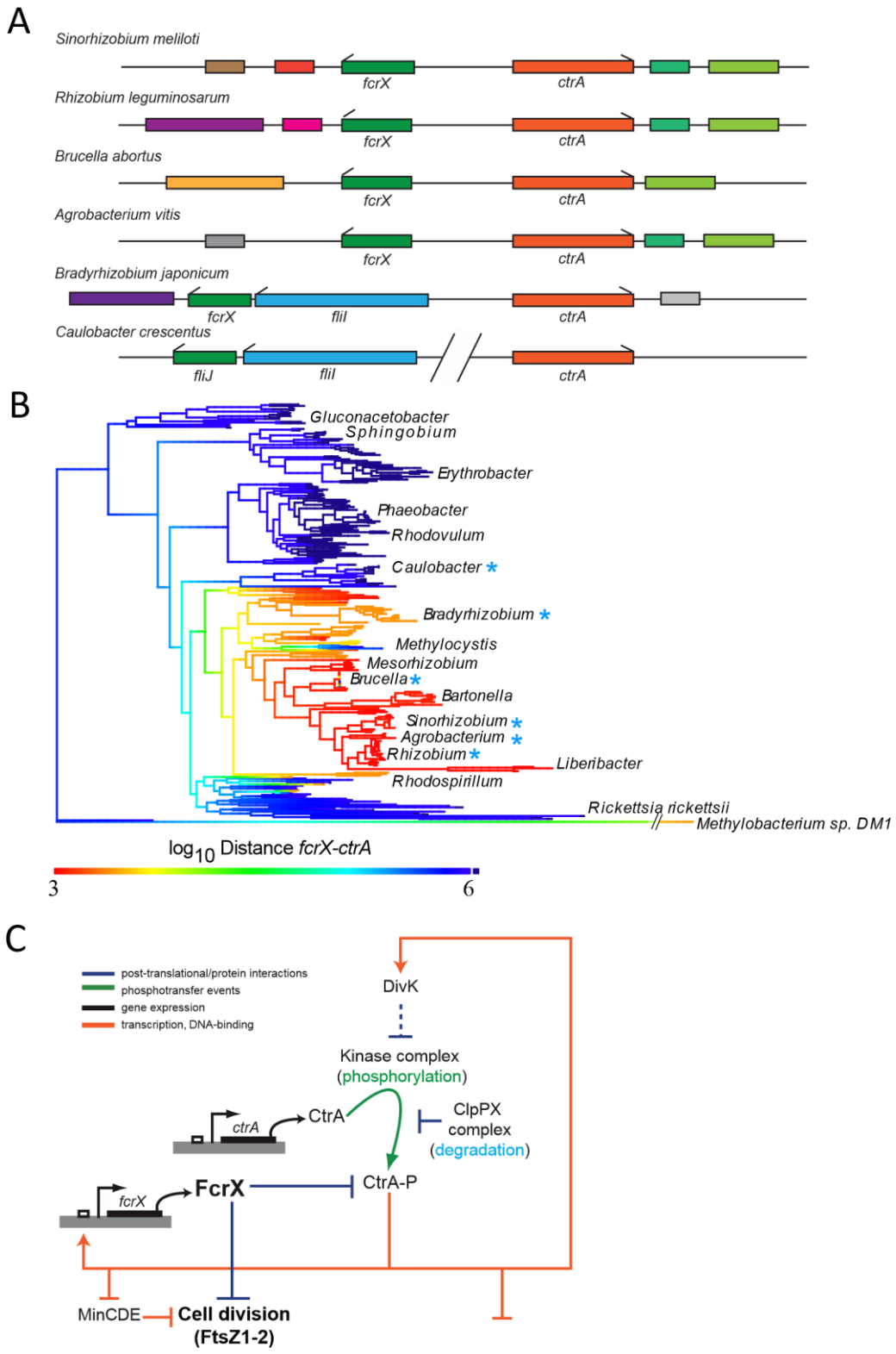


Figure 28: FcrX is conserved among alphaproteobacteria.

(A) Typical organization of *fcrX* genomic loci in model alphaproteobacteria. The presence and the relationship between locations of *fcrX* and *ctrA* genes in those alphaproteobacterial species is highlighted with a blue asterisk in Figure 28B.

(B) Phylogenetic tree of FcrX orthologs-containing species (as described in Materials and Methods). The color code of the tree marks the distance between the *fcrX* and *ctrA* genes (values are bp). Species used in Figure 28A are marked with a blue asterisk, tree based on RecG sequences.

(C) Model of FcrX role in cell cycle regulation with respect to main functions of cell cycle and CtrA/DivK/ClpXP essential regulators.

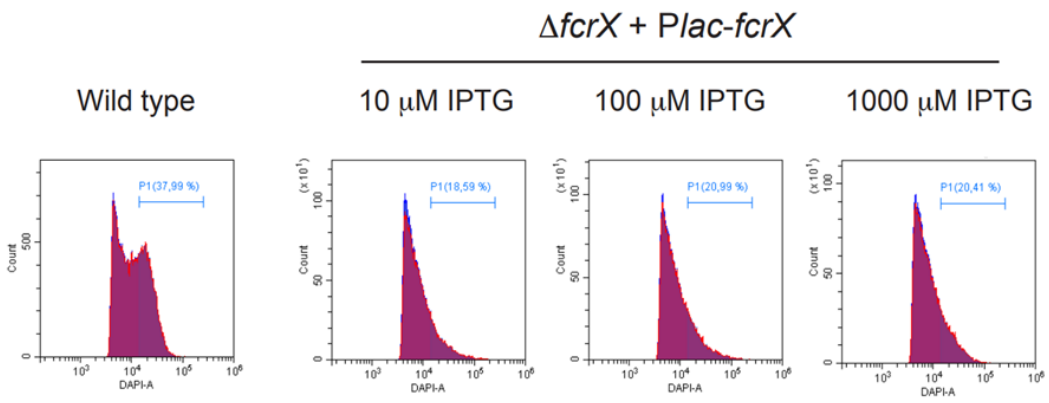


Figure S9: DNA content of bacteroids extracted from 42 dpi nodules determined by flow cytometry as shown in Figure 27A.

Cells were labeled with DAPI.

e- Discussion

In every organism, important functions of the cell are controlled by key factors (master regulators) coordinating many related elements. This is the case, for example, of the cell cycle in eukaryotes, based on cyclins (Wang, 2022), the sporulation process of *Bacillus subtilis*, controlled by Spo0A (Kovács, 2016) and the cell cycle regulation in some alphaproteobacterial, such as *C. crescentus,* where it is controlled by the master regulator signal transduction protein CtrA (Poncin *et al.*, 2018). In this work, we identified in *S. meliloti* a new one key factor of cell cycle and cell division that we named FcrX.

FcrX is a surprisingly small protein with no homology to other, so far characterized, regulators. We predicted that FcrX is an alpha helix-rich protein that can oligomerize, and shares ancestry with a previously characterized chaperon involved in flagellum physiology, named FliJ. In *C. crescentus,* this small protein is specifically required for flagellum functioning through the stabilization of another protein FliI, an ATPase involved in the export of flagellar subunits across the membrane using a dedicated type III protein secretion system (Stephens *et al.*, 1997). The analysis of synteny of FcrX orthologs clearly underlined this association between FcrX and FliJ-FliI, as many orthologs of FcrX are still organized in a *fliIJ* operon. Another striking feature of the analysis of the *fcrX* locus in many alphaproteobacteria is its proximity to the *ctrA* gene. As previously hypothesized, CtrA is considered as an ancestral flagellum regulator in many alphaproteobacterial species, including those in which CtrA also plays a role as cell cycle regulator (Brilli *et al.*, 2010; Greene *et al.*, 2012). This conserved characteristic may suggest that FcrX evolved from FliJ, extending or changing its targets from flagellar components to the divisome protein FtsZ and the cell cycle regulator CtrA.

Indeed, we showed here that FcrX binds directly CtrA in an unknown way and its presence plays a negative role on the steady state levels of CtrA. Conversely, CtrA in addition to many cell cycle genes (Pini *et al.*, 2015) also controls *fcrX* transcription in the second half of DNA replication phase. From this point of view, CtrA-FcrX forms an essential negative feedback loop contributing to the oscillation of CtrA levels during cell cycle (Figure 28C). The presence of an essential genetic negative feedback loop has been also demonstrated in *C. crescentus*, in which DivK and CtrA are the main components of the feedback loop of this species (Biondi *et al.*, 2006). It is tempting to speculate that although the architecture of cell cycle regulation may change between different organisms, CtrA-DivK (linked by a transcriptional relationship) in *C.*

crescentus and CtrA-FcrX in *S. meliloti*, the logical principles behind the regulation remain similar.

In addition, this CtrA-related crucial regulatory function of FcrX is not its only role, as this novel master regulator of cell cycle also negatively controls the main component of the divisome, FtsZ (FtsZ1 and FtsZ2 in *S. meliloti*), by direct protein-protein interaction. However, at this stage it cannot be excluded that FcrX interaction with FtsZ2 (both in BACTH and affinity columns) may involve a ternary complex with FtsZ1 (either from *E. coli* in BACTH or endogenous one in affinity columns) and FcrX, as FtsZ2 lacks a protein interaction domain found in FtsZ1. This dual activity of FcrX makes this factor a novelty in the knowledge of cell cycle regulation in alphaproteobacteria. Although cell division has been shown to be usually regulated by CtrA at the transcriptional level in *S. meliloti*, but also in *C. crescentus* or *B. abortus* (Poncin *et al.*, 2018), this is the first time that a negative regulator of cell cycle is able to connect directly to both cell cycle regulation and the divisome itself. This dual activity of FcrX is responsible of its severe depletion phenotype, leading to a block of cell cycle producing minicells that contain no DNA, but also keeping a mother cell with the genome able to produce new minicells. Considering potential applications that need minicells to perform specific functions (Yu *et al.*, 2021; Kim *et al.*, 2022), the depletion of *fcrX* represents a miniatured minicell factory, which may be exploited in the future for biotechnological purposes.

Another important aspect of FcrX functionality is related to symbiosis and bacteroid differentiation. It has been previously shown that bacteroids in *S. meliloti*, in order to become functionally mature, must eliminate CtrA and FtsZ, leading consequently to elongated cells with multiple copies of DNA. The discovery of a single regulator that is able to control negatively both CtrA and FtsZ suggests that FcrX may play a role during bacteroid differentiation. Indeed, we have shown that FcrX is present in mature bacteroids and its function is required for a correct establishment of symbiosis. Accordingly, a strain constitutively expressing FcrX was able to increase plant biomass with respect to the wild-type situation, suggesting that promoting CtrA and FtsZ downregulation can increase the efficiency of the symbiosis, possibly by predisposing bacteria to terminal differentiation and making this process take place earlier rather than increasing it to a higher level. This will open new frontiers of sustainable agriculture by the use of improved bacterial inoculants based on FcrX deregulation. Even more interestingly, FcrX appears as a conserved factor in several rhizobial species further suggesting that this approach of plant growth amelioration may be extended to other agronomically important legume symbionts.

In conclusion, FcrX is a novel global factor controlling two essential key functions of the cell, regulation of cell cycle progression (CtrA) and cell division (FtsZ) (Figure 28C). This central position and its integrated role in a negative feedback loop with CtrA suggests that cell physiology may rely on FcrX regulation in order to perform higher levels of coordination of cell cycle. In the future, the investigation should move towards exploring how FcrX is regulated and what is the actual mechanism of CtrA and FtsZ1/2 inhibition by FcrX. FcrX indeed represents a small protein with capacities to interact with very diverse targets that may be a tool or a target for antibiotic therapies.

f- Materials and methods

Growth conditions

The strains used in this study are listed in the Table S3. *S. meliloti* 1021 and *E. coli* strains were grown in YEB medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 0.04% $MgSO_4 \cdot 7H_2O$, pH 7.5) at 30°C and LB medium (1% tryptone, 1% NaCl, 0.5% yeast extract) at 37°C, respectively. Media were supplemented with appropriate antibiotics: Kanamycin (50µg/ml), Tetracycline (10µg/ml), Gentamicin (20µg/ml) for *E. coli*, Streptomycin (200µg/ml), Kanamycin (200µg/ml), Tetracycline (2µg/ml), and Gentamicin (20µg/ml) for *S. meliloti*. Depletion strains of *S. meliloti* were grown in a medium supplemented with IPTG (100µM for *fcrX* and 1mM for *ctrA*); the depletion was accomplished by washing three times the culture and resuspending it in a medium lacking IPTG at OD_{600nm} = 0.3. The synchronization experiment was performed as described previously (De Nisco *et al*., 2014).

Strain constructions

The two-step recombination procedure was used to perform the *fcrX* deletion using the integrative plasmid pNPTS138 as previously described (Pini *et al*., 2013). Deletions were verified by PCR using primers flanking the recombination locus (see primers in Table S4).

To construct the fusion between the protein of interest and the fluorescent proteins (CFP or YFP), the Gateway procedure (Thermo Fisher) was used. First, the gene was amplified by PCR (see primers in Table S4) then introduced in the pENTR vector. Then, the vectors were mixed with the destination plasmid carrying the gene coding for the fluorescent protein, to perform the LR reaction as recommended by the manufacturer. The final product was amplified by PCR and cloned in the pSRK vector downstream the P*lac* promoter (Khan *et al*., 2005) and electroporated in *S. meliloti* as previously described (Ferri *et al.*, 2010).

To transduce the *fcrX::tetR* deletion the phage M12 was used (Finan *et al.*, 1984). To do so, the bacteria were grown in LB containing 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ then mixed with the phage to give a multiplicity of infection of 0.5. The mixture was incubated at room temperature for 45 min and subsequently plated on LB plates with the appropriate antibiotics.

In order to identify the *fcrX* promoter, six different *PfcrX-fcrX* constructions were cloned into the P*lac* inducible plasmid pSRK and were electroporated in *S. meliloti* wild type cells. All *S. meliloti* clones containing an extra plasmid-encoded copy of *fcrX* with different promoter lengths were transduced using a phage M12 lysate produced from the strain Δ*fcrX::tetR* + P*lac*-

fcrX and selected in presence or absence of IPTG. To verify the *fcrX* promoter sequence and its regulation by CtrA, the different constructions were introduced in pOTem1 (Meyer *et al.*, 2018) vector using the RF cloning procedure (Ent and Löwe, 2006).

S. meliloti 1021 (wild type) Tn-seq data of the *fcrX* gene during growth in YEB medium was obtained from a previous study (Travin *et al.*, 2023).

Nodulation assays and analysis

M. sativa cultivar Gabès seeds were scarified with pure sulfuric acid for 8 min. After several washes with distilled water, the seed surface was sterilized with bleach (150ppm) for 30 min and seeds were washed again. Finally, seeds were soaked overnight under agitation in sterile water and then transferred onto a Kalys agar plate for one day at 30°C in the dark to allow the germination. The seedlings were planted in perlite/sand (2:1 vol/vol) in 1.5L pots in the greenhouse (24°C, photoperiod 16 h of light and 8 h of dark, humidity 60%) and were inoculated 7 days after planting with 50 ml per pot of the appropriate bacteria at OD_{600nm} = 0.05. Plants were watered every three days, alternating tap water and a commercial N-free fertilizer (Plant Prod solution [N-P-K, 0-15-40; Fertil] at 1 g per liter). Plants were harvested at 6 weeks post inoculation (42 dpi) to analyze bacteroid colonization and nodule development by confocal microscopy, level of bacteroid differentiation by flow cytometry and plant dry mass measurement (Nicoud *et al.*, 2021).

Electron microscopy

Bacteria were prefixed by adding an equal volume of fixative (2% glutaraldehyde in HEPES buffer 200mM, pH 7.2) to the culture medium. After 20 min, the medium was replaced by 1% glutaraldehyde in HEPES buffer for at least 1 h at 4°C. Bacteria were then washed with HEPES buffer, concentrated in 2% agarose (LMP Agarose, Sigma A9414), washed again with HEPES buffer and post-fixed in 1% osmium tetroxide (EMS 19150) for 1h at 4 °C. Samples were washed again in distilled water and treated with 1% uranyl acetate (EMS 22400) for 1 h at 4°C in the dark. Subsequently, samples were dehydrated in a graded series of acetone and embedded in Epon resin. Ultrathin sections (60–90 nm) were cut, stained with uranyl acetate and lead citrate and were analyzed using a Tecnai 200kV electron microscope (field electron interference or FEI). Image acquisitions were made with a digital camera (Oneview, Gatan).

Confocal and wide field microscopy

Nodule imaging was performed on a SP8X confocal DMI 6000 CS inverted microscope (Leica) equipped with hybrid and PMT detectors, a 10x dry (Plan Apo + DIC (NA: 0.4, Leica)) and a 63x oil immersion (Plan Apo + DIC (NA: 1.4, Leica)) objectives. For each condition, multiple z-stacks were acquired (excitation: 405 nm; collection of fluorescence: 415-470 nm for calcofluor excitation: 488 nm; collection of fluorescence: 498-556 nm for Syto9 and excitation: 561 nm; collection of fluorescence: 571-645 nm for PI). Stacks were transformed into maximum intensity projections using ImageJ software.

Time lapse experiments were performed on depleted cells deposited on agarose/YEB (with appropriate antibiotics and inducers) and observed every 10 minutes (up to 16h) on a Nikon Eclipse Ti E microscope equipped with a Yokogawa CSU-X1-A1 spinning disk system.

qRT-PCR experiment

RNA was extracted from bacterial culture samples using Maxwell® 16 LEV miRNA Tissue Kit (Promega). cDNA was produced using random hexamers as primers and the GoScriptTM Reverse Transcription kit from Promega. Amplification of 16S rRNA and *fcrX* cDNA was made using SsoFast EvaGreen Supermix 2X kit (Bio-Rad, France) on a CFX96 Real-Time System (Bio-Rad) instrument and the results were analyzed by Bio-Rad CFX Maestro version 1.1 software (Bio-Rad). For each sample, a biological duplicate was realized. Primers are listed in Table S4.

Flow cytometry

Cells were heated 10 minutes at 70°C and then stained, depending on the experiment, with DAPI (300µM), Syto9 (2.5nM), PI (2.5nM) and Potomac Gold (1mM). After 10 minutes of incubation at room temperature, the cells were processed with Cytoflex bench-top cytometer (Beckman-Coulter) and the data analyzed with CytExpert 2.5 software.

Western blot experiment

The bacterial pellets were prepared and frozen at OD_{600nm} = 0.6. Western blot was performed as previously described (Pini *et al*., 2013). Anti-GroEL are commercial antibiodies against *E. coli* GroEL (Abcam). For anti-FcrX antibodies, *fcrX* was cloned into a pET derivative with N-terminal His6 tag using a Gateway cloning procedure as previously described (Skerker *et al*., 2005). His6-FcrX was purified on a nickel column and rabbits were injected using a 28 days protocol (Pini *et al*., 2013). Purified plasma was then used for Western blots.

Protein interaction experiments

For the Bacterial Two Hybrid (BACTH) experiment (Karimova *et al.*, 1998), the recommendations by the supplier (Euromedex) were applied. To construct recombinant proteins, vectors available from Euromedex were used. These vectors enable the in frame fusion of the proteins subunits of adenylate cyclase from *Bordetella pertussis* (T18, T25) at the C and N terminus. To test protein putative interactions, each appropriate combination of vectors was electroporated into the *E. coli* strain βHT101, deleted of the gene coding for the endogenous adenylate cyclase (*cya* strain). Positive control corresponds to the Tol-Pal from *E. coli* (Wojdyla *et al.*, 2015).

For biochemical protein-protein interaction analysis, a nickel affinity column was used. Cells of *E. coli* BL21 (D3A) expressing His6-FcrX and *E. coli* BL21 (D3A) with no expression vector were induced 3h with 100µM IPTG at 30°C. Cells were harvested, sonicated as previously described (Skerker *et al*, 2005) and soluble lysate of both strains was loaded onto prepacked nickel columns. After several washes of extraction buffer (Tris 100mM, NaCl 500mM, imidazole 30mM, pH 7.5), an *S. meliloti* sonicated lysate was loaded on the columns, washed as previously described, followed by elution at increasing concentrations of imidazole (5%, 10% and 30%), collecting the eluates. Samples were loaded on SDS-PAGE gels and analyzed by mass spectrometry (details in Table S1) or western blot using antibodies direct against CtrA and FtsZ1 or 2 (the same polyclonal antibody is able to detect both copies).

FcrX conservation analysis

Homologs of RecG, FcrX and CtrA were identified by first blast (Camacho *et al.*, 2009) hits, with an e-value cutoff of 10^{-4}. Proteomes for all alphaproteobacteria considered were downloaded from NCBI. Only complete genomes were considered for the analysis. Genomic distances were calculated by using coordinates in the corresponding ".gff" file; the distance dividing two genes was defined as the minimum distance in both directions, *i.e.* taking the circularity of the genome into account. Ancestral state reconstruction of distances was performed and mapped on trees with function contMap from R-package phytools (Revell, 2012). Alignments were performed with Muscle (Edgar, 2004) and refined by hand in AliView (Larsson, 2014); maximum likelihood phylogenetic reconstructions were performed with iqTree (Nguyen *et al.*, 2015), with options -nt AUTO -alrt 1000 -bb 1000, which combines ModelFinder, tree search, ultrafast bootstrap and SH-aLRT test.

g- Acknowledgments

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2- FcrX involvement in the proteolysis of CtrA and FtsZ proteins

a- Foreword

As previously said, the gene *fcrX* is essential and it codes for a small protein (116aa) in *S. meliloti*. The study described in the chapter 1 of the results section, showed its key role in the cell cycle and cell division regulation (Figure 28C). Indeed, FcrX downregulates the accumulation of the master regulator CtrA and both copies of the tubulin-like proteins FtsZ1 and FtsZ2 through a possible direct interaction (Dendene *et al.*, 2023). However, the exact mode of action of FcrX is still to be deciphered. Since the 3D prediction revealed a protein structure with no DNA binding motif and no enzymatic domain prediction, this, consequently implies a possible association with other factors to achieve its function. Thus, the aim of this second part of the thesis is to understand the mechanism by which FcrX induces the downregulation of FtsZ proteins and the master regulator CtrA in cell cycle regulated manner. Moreover, FcrX seems to be essential during the symbiosis process and to be stabilized in the differentiation zone of the nodule, unlike CtrA and FtsZ1/2 (Dendene *et al.*, 2023). Therefore, the understanding of the exact function and a possible link between FcrX and the host plant is intriguing and has to be investigated. Here we propose a potential link between FcrX and the NCR peptides, which are important actors in the terminal differentiation process.

This chapter will be divided into two parts, the first one will deal with the function of FcrX in a free-living cell and the understanding of how FcrX achieves its function, and the second part will focus on the better understanding of FcrX function during symbiosis with *Medicago sativa* by investigating a potential link to the NCR247 peptide. To do so, interaction tests were conducted; the affinity column experiment was realized by the lab before my PhD started. The other experiments such as mutants construction, bacterial two hybrid (BACTH), localization experiments were realized by me and Adam Vieillard, which I supervised during his M1 and M2 internship in the lab. The preliminary experiment in order to investigate the link between the NCR247 and FcrX was realized by me during the last months of my PhD. More experiments are planned to study in depth this topic.

b- Introduction

The bacterial proteins constitute 50% of the cell weight, and are at the base of almost all-biological processes. Thus, their expression has to be tightly regulated. As mentioned previously, the regulation of gene expression can intervene in several levels, in this chapter we will focus on the post-translational level, in particular the proteolysis (Alber and Suter, 2019).

Usually, the proteolysis is achieved by a complex of proteins constituted of a protease and a chaperone (Figure 29). The protease is an enzyme that hydrolyzes peptide bounds linking together the amino acids. The protease can be classified according to their active site residue for example ClpP and HslV are serine and threonine proteases, respectively. The proteolysis process requires an important amount of energy; thus a second protein is involved to power the complex. The second protein is called a chaperone and belongs to the AAA+ protein family that delivers the substrate to the protease in an ATP-dependent manner. For example; ClpX and HslU, are chaperones that associate with ClpP and HslV, respectively.

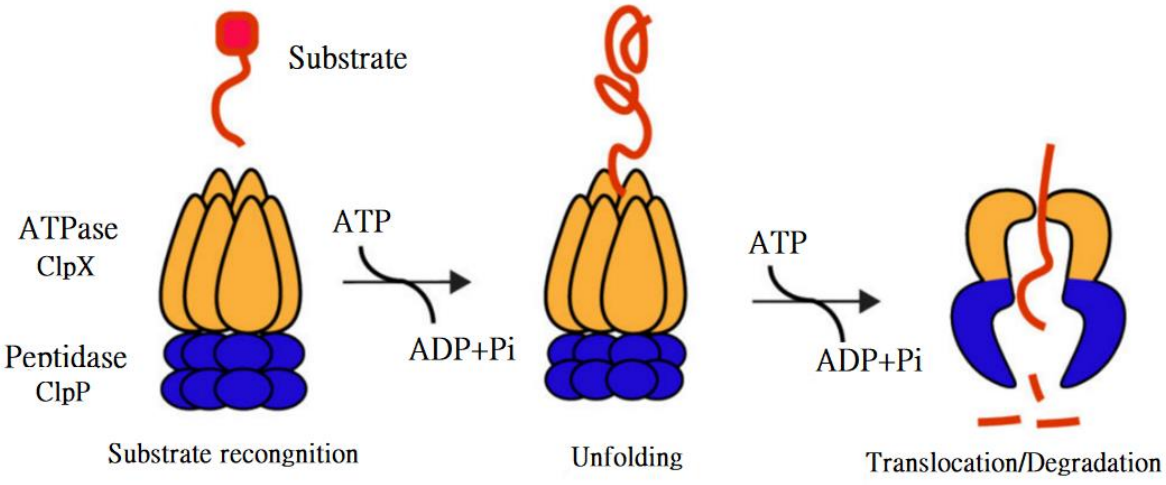


Figure 29: Scheme representing an ATP-dependent protease model such as the conserved ClpXP.

The proteases are composed of an ATP-hydrolysis active unfoldase domain (ClpX in yellow) and a peptidase/protease (ClpP, in blue). The proteolysis substrate is recognized by the protease, unfolded by the ATPase and then translocated into the peptidase to be degraded (Mahmoud and Chien, 2018).

The proteolysis is a fundamental process that allows the maintenance of cell homeostasis. Indeed, the proteolysis is necessary for the degradation of the misfolded proteins to prevent proteotoxic stresses. Moreover, the proteolysis is a prerequisite to allow a constant turnover of protein content and the efficient elimination of proteins that are no-longer useful in the cell, which is essential for oscillatory process such as the cell cycle (Alber and Suter, 2019). The proteolysis is a non-reversible process. Thus, it has to be specific to avoid a random protein degradation. Indeed, the protease complex ClpXP operates on specific proteins. In order to be recognized for the proteolysis, the target proteins usually contain a tag, a short amino acid sequence called degron motif, which is addressed and recognized by the protease to be degraded. For instance, the sequence "AKEPDYLDIPAFLRKQAD" in the C-terminal part of FtsZ in *E. coli* and the degron sequence "DPNEQVNAA" in the N-terminal of CtrA in *C. crescentus* (Izert *et al.*, 2021).

However, other motifs can trigger the proteolysis process, for example, in *Escherichia coli*, the Lon protease is responsible for the proteolysis of approximately 50% of the misfolded proteins implying a mild specificity toward its substrates. When the protein is unfolded, some residues are unveiled which are usually hidden in native protein; therefore the protease Lon is less specific than ClpP in targeting its substrates. In addition, the cell localization of the protease complex is another important driver of the specificity. Indeed, some adaptor proteins are involved in protein delivery to the degradosome. For example, in the alphaproteobacterium *C. crescentus* to ensure the entrance into the S phase, CtrA is degraded by the complex of proteins composed of the protease ClpXP, the adaptors RcdA, CpdR, PopA and the secondary messenger cyclic di-GMP.

The constant expression of ClpXP protease through the cell cycle implies that the other actors are responsible of CtrA oscillatory expression during the cell cycle. This points towards the crucial importance of CtrA subcellular localization in the degradation process. Indeed, the above-cited adaptors are subjected to cell cycle regulation themselves and they drive CtrA and ClpXP localization to the stalked cell pole to promote degradation by increasing the physical contact between the protease and the prey protein (McGrath *et al.*, 2006; Mahmoud and Chien, 2018). In *Sinorhizobium meliloti*, an orthologue of *C. crescentus*, the degradosome factors are conserved except for PopA. Some of these factors are present in multiple copies (three copies of ClpP and two copies of CpdR). It should be noted that ClpP1 and CpdR1 are the copies involved in the cell cycle regulation (Kobayashi *et al.*, 2009; Pini *et al.*, 2013; Ogden *et al.*, 2019) . The operating mode of this degradosome complex in *S. meliloti* is not deciphered yet.

However, we already know that RcdA and CpdR1 are directly involved in CtrA degradation in a cell cycle dependent manner (Kobayashi *et al.*, 2009; Pini *et al.*, 2015).

As explained in the introduction, during the symbiosis process *S. meliloti* undergoes a terminal differentiation program, consisting of an expression profile remodeling, leading to the production of bacteroids. The bacteroids are characterized by an endoreduplication, a cell elongation and a cell membrane permeabilization. This differentiation is governed by the antibacterial NCR peptides produced by the host plant. Indeed, bacterial cells treated with NCR247 *in vitro* showed a bacteroid-like phenotype and an expression profile modification such as a downregulation of CtrA regulon (Van de Velde *et al.*, 2010; Penterman *et al.*, 2014). Moreover, NCR247 directly targets cytoplasmic proteins, among them FtsZ proteins, this interaction leads to a disruption of its stability (Farkas *et al.*, 2014). The absence of FtsZ proteins and CtrA is consistent with the bacteroid phenotype described above. However, NCR targets neither their exact way of function are yet deciphered.

c- Results: unveiling the link between FcrX and the degradosome

To stress on the involvement of FcrX in the proteolysis process of CtrA and FtsZ proteins we realized a Run Off, a technique that allows the measurement of proteins half-life, on depletion strain of *fcrX* in presence and absence of FcrX (the strain construction is described previously in this thesis and elsewhere (Dendene *et al.*, 2023)). Western blot using antibodies against CtrA allowed to measure its half-life. Figure 30 shows an increase of CtrA stability in absence of FcrX expression with respect to the condition were FcrX is not expressed. This result strongly confirms the direct involvement of FcrX in the proteolysis of CtrA and FtsZ proteins.

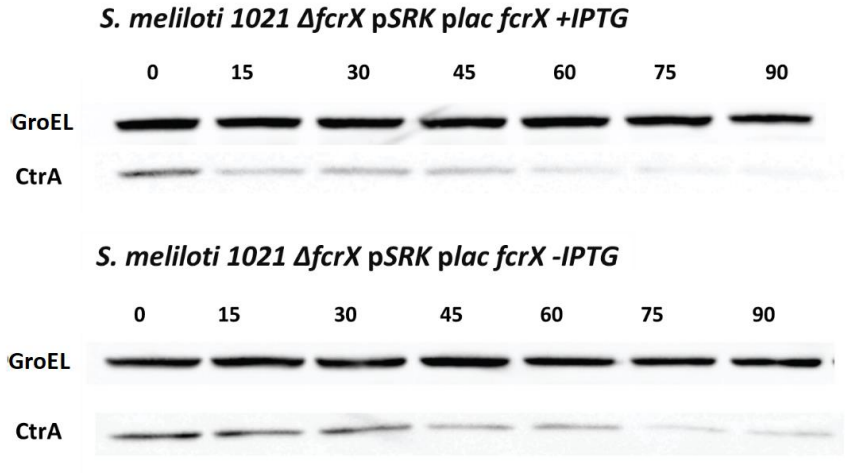


Figure 30: Westen blot representing a Run Off experiment realized on a depletion strain of *fcrX* in presence and absence of the inducer.

Antibodies against CtrA were used to detect the protein and measure its stability. GroEL was used to normalize protein loading and to show protein stability when they are not degraded by the degradosome.

Then, in order to investigate the FcrX mode of action, we analyzed more in details the Nickel affinity column experiment. The results of the mass spectrometry interestingly showed also the presence of the protease ClpP1 (Table S1). The detection of ClpP1 instead of the other two copies of ClpP is coherent, since ClpP1 is involved in the cell cycle regulation just like FcrX. We wanted to confirm the affinity column results, so we realized a BACTH experiment using the *Escherichia coli* model. This experiment is based on the reconstitution of the adenylate cyclase enzyme from *Bordetella pertussis* in presence of interaction between two

proteins. The tested proteins are fused to domains 18 and 25 of the adenylate cyclase and expressed on different plasmids. All the combinations were tested in the *E. coli* strain HB101. As shown in the Figure 31, the results confirmed the previous observations. As explained before, in the well-studied *C. crescentus* model, the proteolysis of the cell cycle factors operates through a degradosome complex. So, we wanted to extend our tests to other factors of the degradosome that may have been missed by mass spectrometry analysis. Thus, we tested the interaction of FcrX with CpdR1 and RcdA, as well as the additional copies of ClpP (ClpP2 and ClpP3). *S. meliloti* contains two copies of CpdR (CpdR1 and CpdR2). However, we chose CpdR1 for our tests, since CpdR1 is the copy involved in the cell cycle regulation as well as the symbiosis (Kobayashi *et al.*, 2009). For ClpP, we decided to test all of the three copies. Despites the clear involvement of ClpP1 in the cell cycle regulation as well as the symbiosis process no conclusive data about the function of ClpP2 and ClpP3 are available. Thus, we though necessary to test the three copies. Moreover, in the bacterium *Mycobacterium tuberculosis* two copies of the protease exist and it was demonstrated that both of them are essential and operates by forming a heptameric rings (Akopian *et al.*, 2012). In addition, *clpP2* and *clpP3* transcription level increases in the fixation zone of the nodule implying their importance in the proteolysis process (Roux *et al.*, 2014c). These tests showed the interaction between FcrX and ClpP1 and ClpX and the two adaptors RcdA and CpdR1. These BACTH interactions, together with previous results with the affinity column, strongly suggest a possible link between FcrX and the degradosome complex. Two hypotheses can be raised from these observations. Indeed, FcrX can constitute a substrate of the degradosome or an adaptor that, in combination with the above-cited adaptor may promote the proteolysis of other substrates such as CtrA and FtsZ proteins (Figure 31). On the other hand, the interaction between FcrX and ClpP2 and ClpP3 gave a negative result. Indeed, the role of these copies of the protease ClpP in *S. meliloti*, is not known yet.

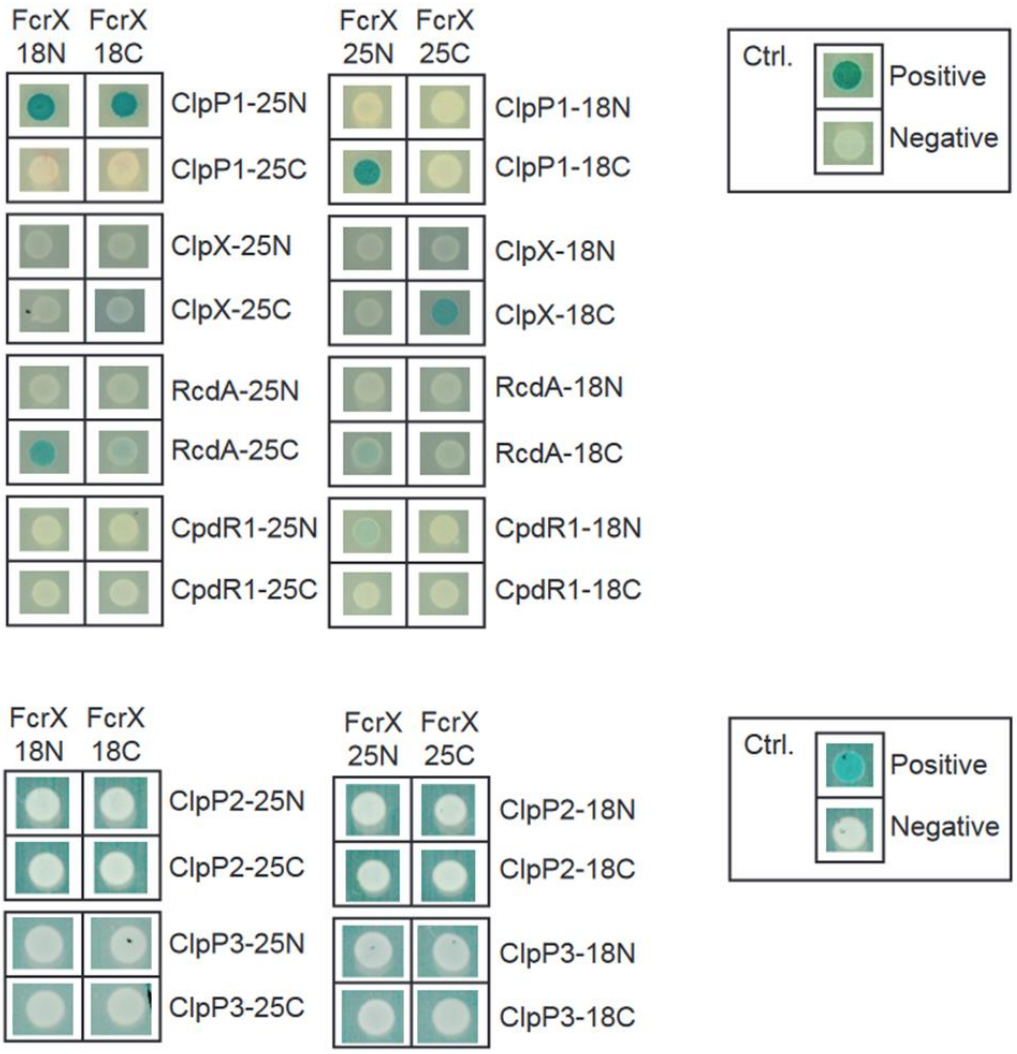


Figure 31: A Bacterial Two Hybrid experiment in the model *Escherichia coli.*

Interaction test between FcrX and the degradosome factors ClpP1, ClpP2, ClpP3, ClpX, RcdA and CpdR1. Upper right box shows negative and positive controls provided by the supplier (see Materials and Methods in Dendene *et al.*, 2023).

Subcellular localization of ClpXP was determined by constructing a CFP fusion at the C-terminus of both the protease ClpP1 and the unfoldase ClpX. Both of these constructions were expressed on a pSRK plasmid under the control of a P*lac* promoter and introduced in a wild type strain of *S. meliloti*. The microscopy observations were realized using a wide field microscope on an asynchronous culture supplemented with IPTG (1mM and 100µM IPTG for ClpP1-CFP and ClpX-CFP, respectively) to induce the expression of the tagged protein. These observations showed a polar and a mid-cell localization of both proteins ClpP1 and ClpX

(Figure 32). This localization is consistent with the available data from *C. crescentus*, where the localization of the proteasome ClpXP was already demonstrated. Indeed, in *C. crescentus* the proteasome localizes at the stalked pole during the G1-S cell cycle transition for CtrA degradation and at the mid-cell in the predivisional phase (Iniesta and Shapiro, 2008; Smith *et al.*, 2014). This result suggests the conservation of the same pattern in *S. meliloti*.

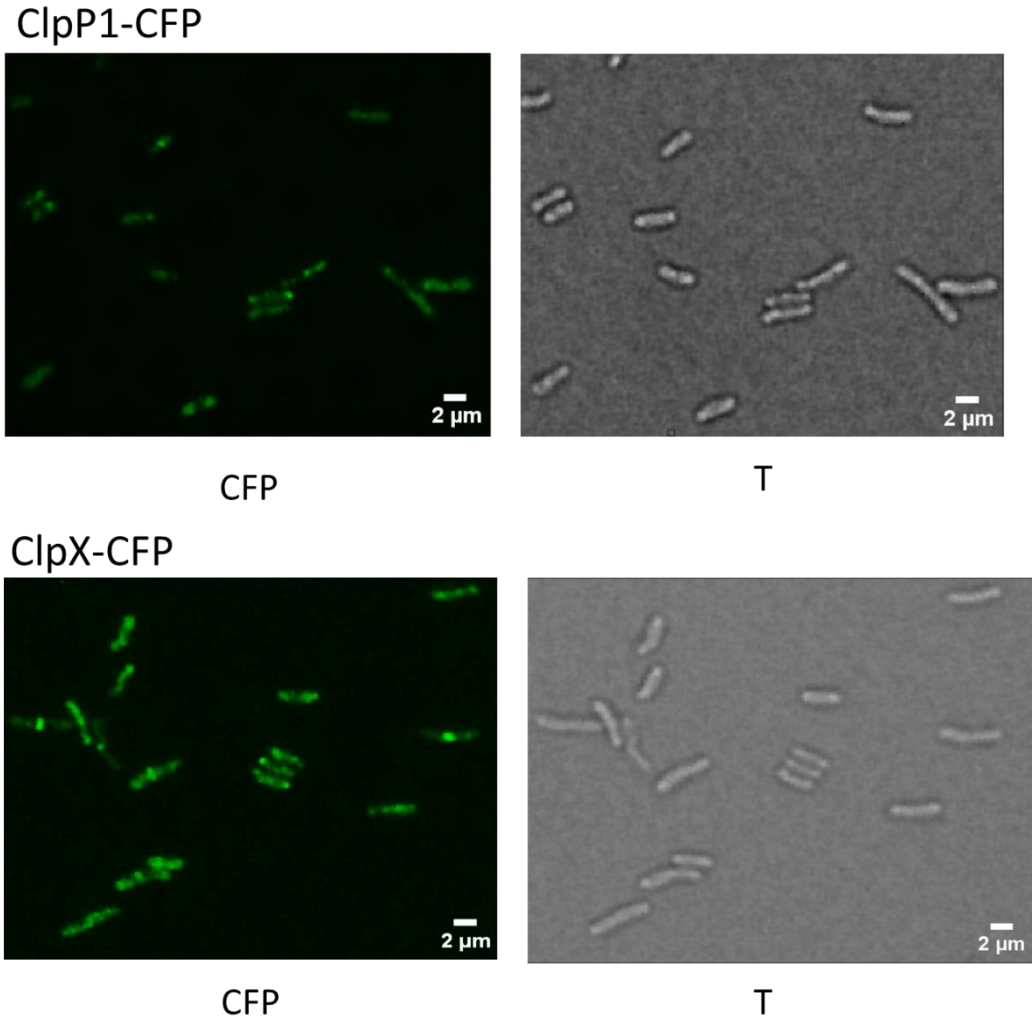


Figure 32: Subcellular localization of Clp1 and ClpX in *S. meliloti.*

Microscopy images showing the subcellular localization of ClpP1-CFP and ClpX-CFP fusions at the cell pole of *S. meliloti* CFP and T refers to epifluorescence and transmission acquisition, respectively. The scale bar is 2µm.

Such as the master regulator CtrA, the regulators involved in the cell cycle regulation are commonly subject to proteolysis by the degradosome complex. To verify if FcrX follows the same pattern, we realized a Run off experiment on wild type strain of *S. meliloti* and measured the FcrX and CtrA stability. To do so, we treated a wild type culture with the translation inhibitor chloramphenicol in order to stop the translation process and we took samples each 15min up to 75min. As expected, CtrA concentration, which is known as a degradosome target, drops after 15min from the beginning of chloramphenicol treatment (Figure 33). Oppositely, FcrX concentration remains stable through the timeline of a cell cycle. This result strongly suggests that FcrX does not constitute a target of proteolysis. Considering that FcrX interacts with ClpP1 and ClpX (Figure 31) and that it is not degraded by the degradosome (Figure 33). It is tempting to speculate that FcrX could play a role of a new class of adaptors between the degradosome (ClpXP) and its substrates (CtrA, FtsZ1 and FtsZ2).

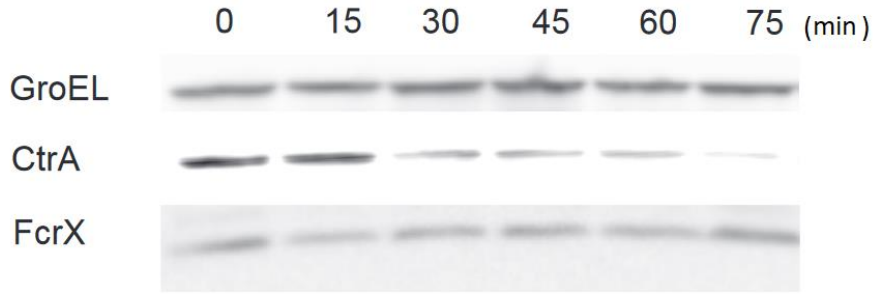


Figure 33: Protein stability of CtrA and FcrX.

Western blot representing a Run Off realized over 75 minutes after addition of chloramphenicol on a wild type exponential culture of *S. meliloti*. Antibodies against CtrA and FcrX were used to detect the proteins and measure their stability compared to GroEL chaperone (control for a protein that is not subjected to degradation by ClpXP).

We wanted to strengthen these observations, so we realized a Run off on a depleted strain of *rcdA* and we compared the FcrX and CtrA stability in presence and absence of RcdA expression. As observed previously in the wild type strain, FcrX remains stable in both conditions while we observe a clear effect of RcdA on CtrA stability. In absence of RcdA expression, CtrA stability increases (same pattern was observed for CpdR1) (Pini *et al.*, 2015)(Figure 34). However, as mentioned before, the degradosome complex is organized

hierarchically and some adaptors are specifically able to degrade specific proteins. For example, RcdA is added to the degradosome chain to specifically target CtrA proteolysis. Thus, the absence of stability variation in absence and presence of one of the adaptors is not enough to demonstrate the inefficiency of the degradosome on FcrX. However, it is important to explore all eventualities to reinforce our results. These results strongly suggest the involvement of FcrX as an adaptor in the proteolysis of CtrA and possibly FtsZ1 and 2.

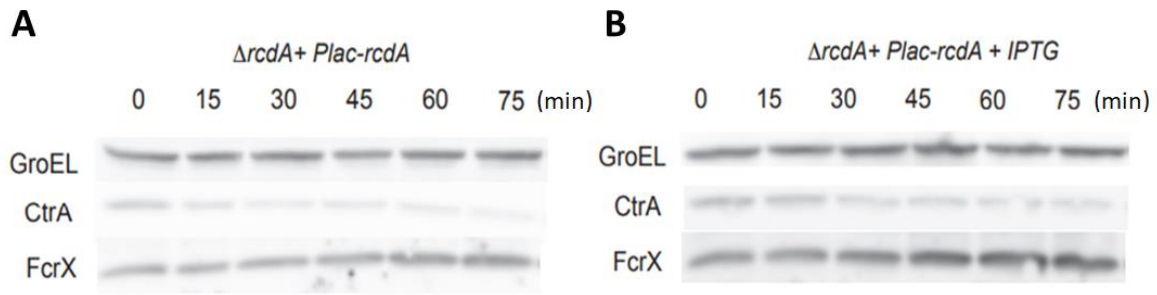


Figure 34: Protein stability of CtrA and FxrX in a deleted backround of *rcdA*.

Western blot representing a Run Off realized on a depletion strain of *rcdA* in presence (B) and absence (A) of the inducer IPTG. Antibodies against CtrA and FcrX were used to detect the proteins and measure their stability. GroEL was used as a control protein that is not a substrate of the degradosome.

FcrX involvement in the molecular development of symbiosis

As showed in the previous chapters, the cell cycle of *S. meliloti* is tightly linked to the symbiosis process. Indeed, to permit a cell differentiation of the symbiont into a bacteroid, the cell cycle must be misregulated, notably by downregulating the accumulation of CtrA and FtsZ proteins (Pini *et al.*, 2013; Penterman *et al.*, 2014; Farkas *et al.*, 2014). However, as explained in the first chapter of this thesis the mechanism by which these proteins disappear in the bacteroid is not known yet. The NCR peptides are the host factors inducing this cell differentiation. Indeed, *S. meliloti* cells treated with NCR peptides *in vitro* reproduce the bacteroid phenotype and, among NCR targets, CtrA and its regulon as well as FtsZ proteins were identified. Since the disappearance of this latter is a prerequisite for the symbiosis

establishment, it is reasonable to propose FcrX as an attractive unique target for the host plant regarding its negative regulation on those factors.

To explore the potential link between FcrX and the NCR peptides, a sensitivity test toward the NCR247 was realized on a depletion strain of *fcrX*. The depletion strain was grown with different concentration of the inducer (0, 10 and 100 µM IPTG), in order to obtain different levels of FcrX expression, and then cultured in a medium supplemented with increasing concentration of the NCR247 peptide (50, 25, 12.5, 6.25 and 0 µg/ml). These cultures were compared with a wild type strain treated with the same concentration of NCR247. The Figure 36 shows an absence of growth for all the conditions treated with the highest concentration of the NCR247 peptide (50µg/ml) with respect to the condition grown without NCR247 (Figure 35A, B, C and D). The wild type strain treated with (25µg/ml) shows a growth delay around 30 hours, while the conditions treated with the lowest concentrations of NCR247 were able to recover a similar growth compared to the non-treated one, approximately at the same time (Figure 35A). For the depletion strain of *fcrX* grown with 100µM IPTG, no growth is observed in presence of 50µg/ml nor 25µg/ml. In presence of 12.5µg/ml and 6.25 µg/ml of NCR247 a growth recovery is noticed around 30 hours and 15 hours, respectively (Figure 35B). The same behavior is observed for the depletion strain of *fcrX* grown with 10µM IPTG (Figure 35C). A growth delay in presence of the NCR is noticed between the wild strain and the depletion strain of *fcrX* grown in 100 and 10µM IPTG. This result can be explained by the variation of FcrX amount induced by the different concentrations of IPTG. The different levels of FcrX expression modulate the amount of NCR247 targets CtrA, FtsZ1 and FtsZ2. Thus, when CtrA, FtsZ1 and FtsZ2 decrease in the cell, the effect of the peptide on the bacteria is enhanced. This experiment shows evidence that FcrX interferes with the action of NCR247. However, it still not sufficient at this level to deduce the nature of this link.

Finally, the depletion strain of *fcrX* grown in absence of the inducer (Figure 35D), shows as expected a growth defect even in absence of the peptide, interestingly, in presence of 25µg/ml a growth recovery is noticed around 50hours. This can be explained by the opposite phenomenon noticed previously. The absence of FcrX induces an accumulation of CtrA and FtsZ proteins in the cell, which may temperate the effect of the NCR247 on the bacteria (Dendene *et al.*, 2023). However, this result has to be reproduced in the future to exclude the possible isolate effect.

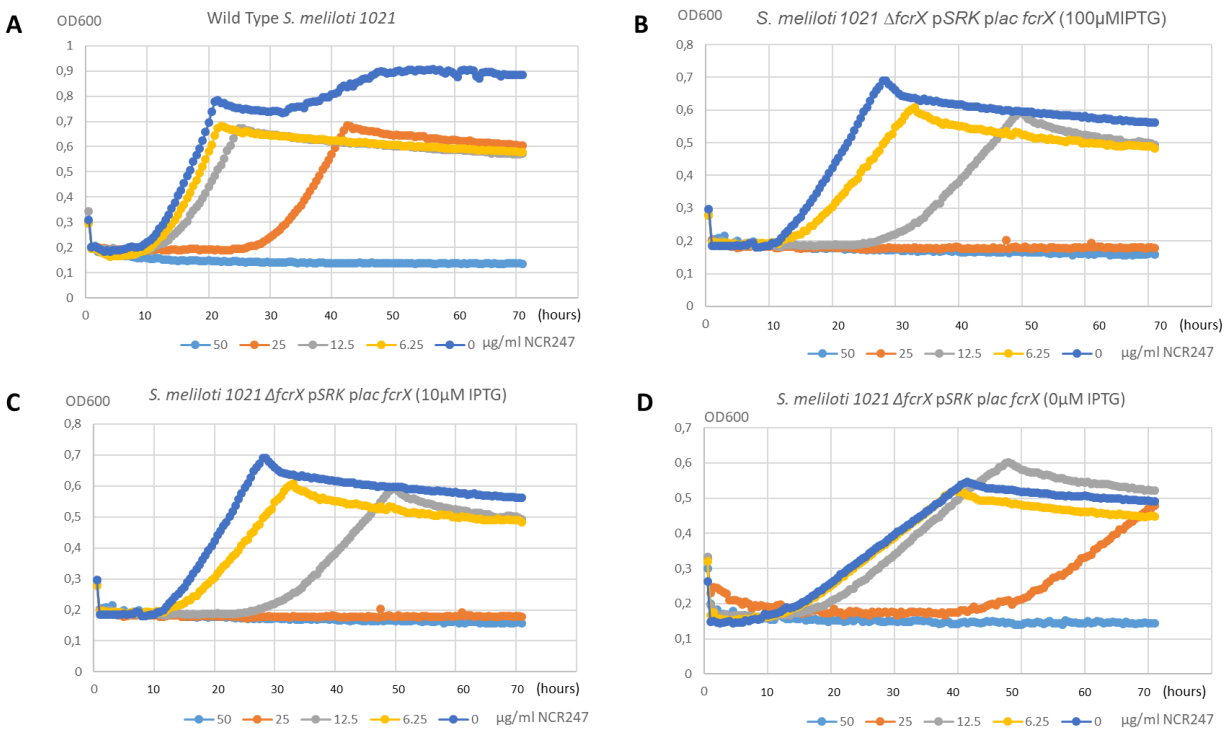


Figure 35: Sensitivity test of *S. meliloti* wild type and *fcrX* depletion strains toward the peptide NCR247.

(A) Growth curves of *S. meliloti 1021* grown in presence of a serial dilution of the NCR247 (50, 25, 12.5, 6.25 and 0 µg/ml). The culture medium was supplemented with 100µM (B), 10µM (C) or without (D) IPTG and the *fcrX* depletion strain was grown in presence of a serial dilution of the NCR247 (50, 25, 12.5, 6.25 and 0 µg/ml).

d- Materials and Methods

Strains and media

The strains used in these experiments are listed in the Table S5 (Annex). *S. meliloti* 1021 strains were grown in YEB medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 0.04% $MgSO_4 \cdot 7H_2O$, pH 7.5) or Minimal medium (0.5% MOPS-KOH buffer, 0.49% NH_4Cl, 0.0875% NaCl, 0.05% KH_2PO_4, 0.05% $MgSO_4$, 0.0125% $CaCl_2$, 0.0125% Biotin, 0.0001% $CoCl_2$, 0.095% $FeCl_3$, 0.25% glucose) at 30°C and *E. coli* strains were grown in LB medium (1% tryptone, 1% NaCl, 0.5% yeast extract) at 37°C. Media were supplemented with appropriate antibiotics: kanamycin (50µg/ml), tetracycline (10µg/ml), gentamicin (20µg/ml) for *E. coli*, streptomycin (200µg/ml), kanamycin (200µg/ml), tetracycline (2µg/ml), and gentamicin (20µg/ml) for *S. meliloti*. Depletion strains of *S. meliloti* were grown in a medium supplemented with IPTG (100µM for *fcrX* and 1mM for *ctrA*); the depletion was accomplished by washing three times the culture and resusp S(ending it in a medium lacking IPTG at OD_{600nm} = 0.3. The strains of *S. meliloti* containing a protein fused with a fluorescent protein were grown in a medium supplemented with IPTG to induce the expression the tagged protein.

The method used to construct the fusion between the protein of interest and the fluorescent proteins (CFP) and the strain used for the BACterial Two Hybrid (BACTH) experiment is described in (Dendene *et al.*, 2023).

The primers used in this chapter are listed in table S6 (Annex).

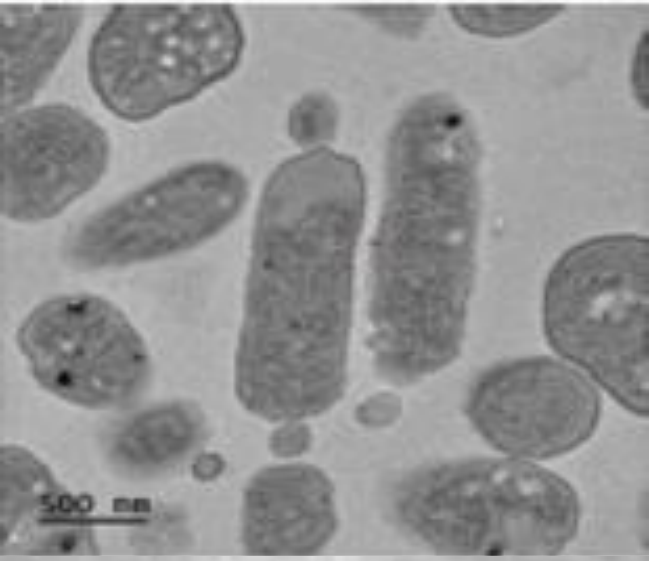
Western blot and Run Off experiments

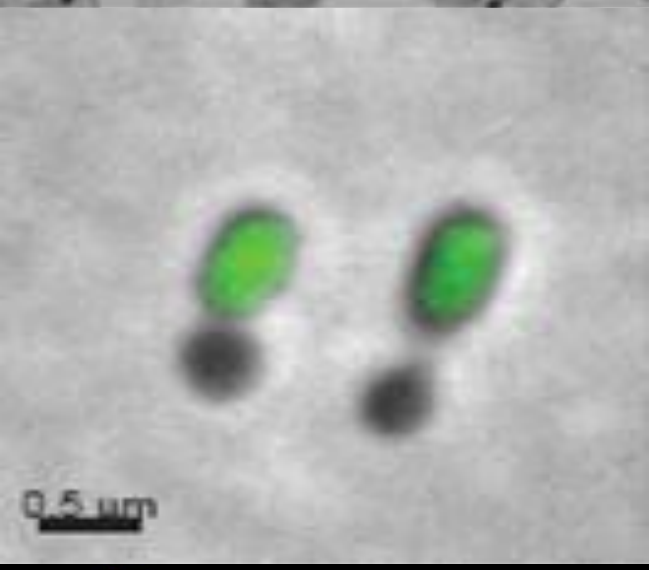
A Run off experiment was realized to measure the proteins stability. To do so, 100µg/ml of chloramphenicol was added to an exponential culture DO600nm = 0.6 in order to stop the translation process. Samples were taken each 15min during 120min. A Western blot using antibodies against FcrX, CtrA and GroEL was realized on the samples as previously described (Dendene *et al.*, 2023).

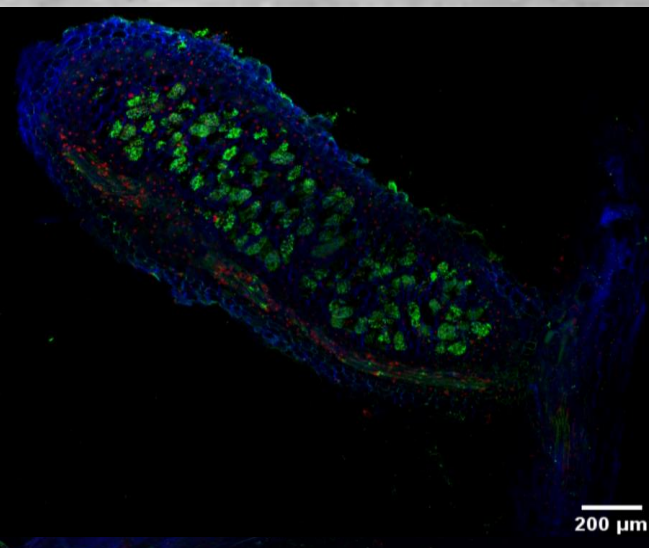
Sensitivity tests

Bacterial cultures were grown to exponential phase and diluted to OD_{600nm} = 0.05 in minimal medium supplemented with the appropriate antibiotics and the NCR247. The NCR247 mature peptide coding sequence from *Medicago truncatula*, was synthetized by ProteoGenix.

Several concentrations of this peptide were tested (50µg/ml, 25µg/ml, 12.5µg/ml and 6.25µg/ml), a culture without NCR peptide was used as a reference. The cultures were transferred to a Falcon 96-well Clear Flat Bottom plate. A TECAN SPARK plate reader was used to monitor every 10 minutes the bacterial growth for 48h at 30°C and 200rpm.







General discussion

a- The discovery of a new cell cycle regulator

As discussed in the article (first part of results), entitled "*Sinorhizobium meliloti* FcrX coordinates cell cycle and division during free-living growth and symbiosis", FcrX occupies a key role in the *S. meliloti* cell cycle regulation. Indeed, the *fcrX* depletion phenotype shows a strong cell cycle defect, which already suggested its implication in this biological process. Thus, a Western blot experiment together with the protein-protein interaction tests showed a negative effect of FcrX on the master regulator CtrA and the FtsZ proteins (FtsZ1 and 2) by, as shown by several techniques, a potential direct interaction. Interestingly, these results place FcrX at the junction point of two separate processes; the DNA replication (inhibited by CtrA) and the cell division (activated by FtsZ1 and possibly 2). Indeed, FcrX seems to be the intermediate of the principal protagonists of these two latter phenomenon CtrA and FtsZ proteins for their degradation. The DNA replication and the cell division are concomitant and indirectly linked in other organisms. For example, in *C. crescentus* CtrA is degraded by the degradosome composed by ClpXP, CpdR, RcdA and PopA, while FtsZ is degraded by ClpXP and ClpAP but, to the best of our knowledge, it does not involve adaptors. This observation may raise the hypothesis that FcrX, just as global regulators, may provide a more efficient and rapid response by simplifying the network regulation. This phenomenon is commonly observed in mutualistic interactions, the symbiont in this case tends to simplify the regulation circuit until it turns into an obligate symbiont such as the bacterium *Buchnera spp.* and the pea aphid (Pontes *et al.*, 2011). However, even if this hypothesize is attractive, it should be noted that this evolutionary pathway is not easy to apply for *S. meliloti* and *M. sativa*. Indeed, the prerequisite to switch from a facultative to an obligate endosymbiont are still blurry (Coba de la Peña *et al.*, 2017).

The phylogenetic and functional study among proteins with similarities to FcrX revealed that FcrX function is conserved in the *Rhizobiales*, for example in *Brucella abortus* and *Agrobacterium vitis*. On the contrary, its more distant homolog FliJ from *C. crescentus* is not functionally able to complement FcrX deletion, as it is a protein esclusively implicated in biogenesis of the flagellum. Interestingly, FcrX orthologs are essential genes and coded closest to *ctrA* gene, while the deletion of *fliJ* is viable in *C. crescentus* and the genomic distance from CtrA is greater compared to the orthologs. The main difference between *C. crescentus* and both *B. abortus* and *A. vitis* is its lifestyle. Indeed, *C. crescentus* is a free-living aquatic bacterium while *B. abortus* and *A. vitis* are human pathogen and phytopathogen, respectively, implying that at a certain point of their life, these bacteria will interact closely with a eukaryotic host. We

can speculate from these observations that bacterial species physically interacting with host organisms possess a different regulation network, which seems to be more simplified.

Furthermore, we could suggest the presence of an evolutionary link between FcrX and FliJ. Indeed, FliJ could be the ancestral version of FcrX that acquired novel functions related to the cell cycle regulation, thus, to permit the adaptation of the organism to new environments. This pattern was also observed for CtrA which possess an ancestral function limited to the regulation of cell motility and chemotaxis which acquired from an evolutionary point of view the function of cell cycle regulation (Brilli *et al.*, 2010). Hence, it would be interesting to investigate a residual conserved potential role of FcrX still in the *S. meliloti* motility and chemotaxis. Moreover, a functional study of FcrX and its homologs in a larger group of species and larger scale of phylogenetic analysis would help understanding how it acquired its cell cycle related functions. Finally, the existence of such central regulator of FcrX may represent a reasonable therapeutic target. This could help to prevent pathogen proliferation by directly triggering the disruption of the cell cycle and cell division. To do so, a screening of molecules that could target directly FcrX or its orthologues from pathogen species, by sequestration may prevent its activity and consequently block the cell cycle progression.

The *fcrX* depletion leads to the production of small cells called "*minicells*", empty of DNA. This phenotype is consistent with the overproduction of the master regulator CtrA (DNA replication arrest and cell division continuous activation) and FtsZ1, 2 (Z ring formation). This minicell phenotype is stable, which implies a specific function of FcrX toward CtrA and FtsZ proteins. In addition, this phenotype reminds us of the Δ*minCDE* from *Escherichia coli.* Indeed, the deletion of the *min* system in *E. coli* leads to the misplacement of the Z ring, this, resulting in the production of small cells empty of DNA (Adler *et al.*, 1967; Corbin *et al.*, 2002). Interestingly, CtrA inhibits the transcription of the *minCDE* system in *S. meliloti*, which explains the phenotype similarity in these two different species. The stability of the minicell phenotype in *S. meliloti* may represent an interesting tool in therapeutic field. Hence, in the context of expression of antigens in a pathogen free system, the proteins of human pathogens from alphaproteobacteria, such as *B. abortus*, *Bartonella* spp. or *Rickettsia* spp. could be expressed in the FcrX depletion strain of *S. meliloti,* in order to express the extracellular proteins on the minicells' envelope. Thus, these minicells presenting antigens at their surface could be used as triggers of the human immunity response as previously described (Farley *et al.*, 2016).

b- FcrX transcriptional regulation by CtrA

The qRT-PCR as well as the Western blot experiments realized on a depleted strain of *ctrA*, showed a decrease of FcrX in the absence of CtrA. In addition, the identification of the promoter sequence of *fcrX*, allowed us to reveal the presence of an active CtrA box. It was demonstrated that this latter is involved in the positive activation of *fcrX* transcription. These results point to the presence of a negative feedback loop between CtrA and FcrX, where CtrA positively activates the transcription of FcrX and, in turn, FcrX regulates negatively the accumulation of CtrA. This kind of regulation circuits implying an ON and OFF switch of gene expression, is the cornerstone of oscillatory processes such as cell cycle and adaptive responses to the external stimuli (Pigolotti *et al.*, 2007). It is also interesting to draw the parallel between the negative loop linking DivK and CtrA in *C. crescentus* with FcrX and CtrA in *S. meliloti*. These two negative feedback loops are different but both lead to the negative posttranslational regulation of CtrA. Indeed, the logic regulatory system in *C. crescentus* could be widely conserved among alphaproteobacteria. However, notable differences in the system connectivity and regulator proteins can be observed in different species. This selection of different regulation strategies can be explained by the ecological niches and lifestyle diversity of these species (McAdams and Shapiro, 2011).

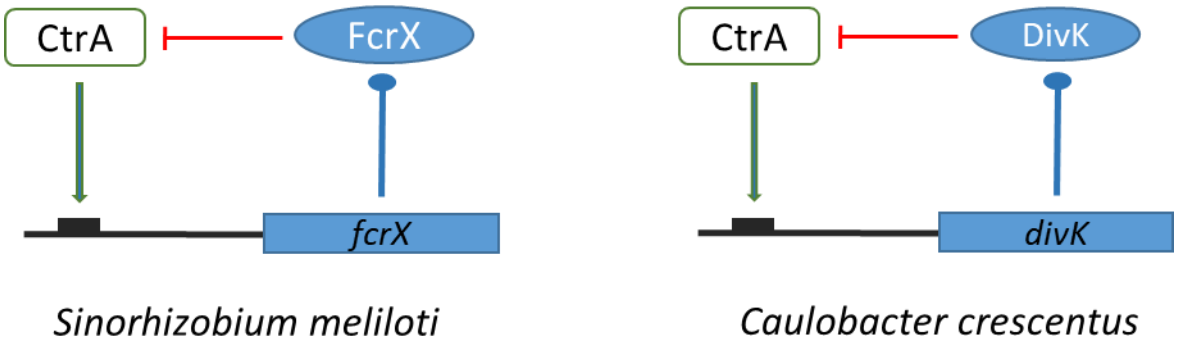


Figure 36: Comparisons of CtrA regulation network between *Caulobacter crescentus* and *Sinorhizobium meliloti.*

Scheme representing two negative feedback loops between CtrA and DivK in *C. crescentus,* and between CtrA and FcrX in *S. meliloti.*

The promoter sequence identification showed a 5'-UTR sequence of *ca.* 200 bp upstream the coding sequence of *fcrX*. The presence of a long untranslated sequence may

suggest a possible post-transcriptional regulation of *fcrX*. The involvement of the 5'-UTR in the post-transcriptional regulation in bacteria is well documented (Picard *et al.*, 2009). Indeed, this untranslated region can represent a reservoir of regulation mechanisms, such as, riboswitches, binding sites for sRNA or even temperature-sensitive sequences that can change their conformation to promote or not the translation of the downstream coding sequence (Menendez-Gil and Toledo-Arana, 2020). Hence, it would be interesting to explore this hypothesis that may uncover an additional layer of *fcrX* regulation. Moreover, sRNA involved in the cell cycle regulation were already identified in *S. meliloti*. Indeed, several interesting cases of ncRNAs regulating cell cycle have been discovered in *S. meliloti*. For example, the sRNAs EcpR1 post-transcriptionally repress the expression of *dnaA* and *ctrA*, while GspR represses that of *ctrA* (Robledo *et al.*, 2015; Robledo *et al.*, 2018).

c- FcrX subcellular localization

The subcellular localization of FcrX was investigated by constructing a translational fusion of FcrX with a Yellow Fluorescent Protein (YFP). This construction was expressed in a *fcrX*-deleted background, suggesting that tagging FcrX with a fluorescent protein doesn't compromise its essential function at least in free living conditions. This experiment showed a mid-cell localization of FcrX in pre-divisional cells and as well as a cell pole localization in the daughter (smaller) cell. As discussed in the result chapter (article submitted), this localization pattern is consistent with the function of FcrX. Accordingly, the same translational fusion with the target proteins showed a mid-cell localization for FtsZ proteins and the degradosome factors ClpP1 and ClpX, and a cell pole localization for CtrA. Furthermore, we established a link between localization and the phase in which FcrX should be the most active. In fact, the Western blot and qRT-PCR experiments realized on a synchronized culture of the *S. meliloti* wild type strain, showed a distinct increase of FcrX expression at the late S-phase. This is consistent with the previous result demonstrating the transcriptional activation of *fcrX* by CtrA. Hence, FcrX seems to be expressed and localized in a cell cycle phase dependent manner in the second part of S-phase, this feature is commonly observed with cell cycle regulators, which are submitted to a spatiotemporal regulation (Laloux and Jacobs-Wagner, 2013).

For the first time of CtrA studies, a fully functional fluorescent version of CtrA was created in *S. meliloti.* This translational fusion of CtrA with Cyan Fluorescent Protein (CFP) at the C-terminus was made functional by adding a 15 amino acid tail, which corresponds to the degradation tag recognized by the protease in a *ctrA*-deleted background. This construction has

never been developed, even in the model organism *C. crescentus*. Thus, this construction will allow us in the future to study with more confidence the localization dynamics of CtrA in *S. meliloti* and possibly in other related organisms. However, this type of construction does not permit the native oscillatory expression of the gene as the native promoter has been replaced. Thus, in order to keep this feature, we could express this fusion under the control of the *ctrA* promoter instead of the P*lac* promoter.

d- FcrX 3D structure prediction

As previously said in this thesis, FcrX represents a new class of cell cycle regulators with many aspects still unknown, including its 3D structure. The 3D prediction of FcrX protein structure using AlphaFold2 revealed an alpha helix-rich lacking DNA-binding domain neither enzymatic motif. These results suggest that FcrX does not downregulate the expression of CtrA and FtsZ proteins by itself but it implies other mechanisms. Moreover, the affinity column and BACTH tests showed a direct interaction with the degradosome giving more insight about FcrX mode of action. Indeed, regarding these observations, FcrX seems to fulfill all the criteria as an adaptor of the degradosome; it interacts directly with the targets and the actors of degradosome, it localizes with these interactors and downregulates their accumulation. Interestingly, the degradosome is much less characterized in the *S. meliloti* compared to the model *C. crescentus*. In this latter species, it is known that the multiple factors are implicated in hierarchical and ordered manner for a specific degradation of CtrA (Joshi *et al.*, 2015). As seen before, the same degradosome actors are present in *S. meliloti* in the exception of PopA, although RcdA and CpdR1 are involved in CtrA degradation. Thus, we could speculate about the conservation of the same pattern in *S. meliloti* with respect to some differences. Indeed, the run-off experiment clearly showed that FcrX does not constitute a degradosome target, implying that the interaction observed between FcrX and the members of the degradosome is rather due to the involvement of this latter in the degradation of CtrA and FtsZ proteins than for its own degradation. From this observation, we can speculate that FcrX acts as an adaptor that links both CtrA and FtsZ1/2 to the degradosome and may be a factor that recruits the target proteins to the cell pole to permit their degradation. As mentioned before, PopA is only present in *C. crescentus* and its closest species (Brilli *et al.*, 2010). We could make the parallel with FcrX, which like PopA is present in a limited range of genera from the order of *Rhizobiales*. However, these two factors do not share any suqesuence homology, but the presence of FcrX despite the absence of PopA may suggest the need for multiple adaptors in order to regulate CtrA. However, as a perspective we

could investigate the possible resemblance between PopA and FcrX at the structural level, using the alphafold approach.

In addition, in *C. crescentus*, the ClpXP complex punctually localizes to the mid-cell to degrade FtsZ and at the cell pole for CtrA degradation. Our results, in *S. meliloti* suggest that ClpXP1 behave in the same manner. Indeed, ClpP1 showed a slight cell pole and mid-cell localization while a distinct cell pole and mid-cell localization was observed for ClpX. These observations stress on the previous conclusions about the degradation pattern conservation between these two organisms.

In the frame of a funded grant by the National Reaserche Agency (ANR), with biochemists from Pasteur institute, biochemical and bio structural experiments are ongoing in order to test *in vitro* the dimerization of FcrX and its interactions with CtrA and FtsZ. Moreover, the degradosome complex components could be purified and tested in order to reconstitute the role of the potential chain of adaptors, including FcrX, during the degradation of a target protein(s).

e- FcrX during symbiosis with the legume plant *Medicago sativa*

The infection of the legume plant *Medicago sativa* with *S. meliloti* strains expressing lower levels of FcrX showed a symbiosis defect, suggesting that, as in free-living conditions, FcrX expression may be important in a symbiotic context. This observation may be linked to a direct involvement of FcrX in the symbiosis process, or it may be the result of a general fitness loss, which could affect the inner physiology of the bacteria and therefore prevent them to fulfill the nodule colonization. Moreover, the strain used in this experiment expresses FcrX in a constitutive manner, which is not an accurate reproduction of the oscillatory expression observed in a wild type condition. This may be detrimental for the establishment of the symbiosis. As observed with CtrA, a constant presence of this latter is lethal for the cell, which stresses on the essentiality of the oscillatory expression of regulators implicated in the cell cycle process. To solve this problem, we could introduce different constructs of *fcrX* under the control of its native promoter (leading the different expression levels), while preserving its oscillatory behavior.

Interestingly, a Western blot realized on bacteroids showed the presence of FcrX, which as expected was not the case for CtrA and FtsZ proteins. Moreover, transcriptomic analysis realized by Roux *et al.* (2014) showed an absence of *fcrX* transcription in the bacteroid, which is consistent since CtrA the transcriptional activator of *fcrX* is absent in the bacteroid. Thus, the

presence of FcrX in the bacteroid could be the result of the protein inherent stability. This result may also suggest the implication of an additional factor from the host that may stabilize FcrX regarding its negative regulation toward CtrA and FtsZ proteins.

As mentioned in the introduction, the terminal differentiation of *S. meliloti* is a prerequisite for an efficient symbiosis establishment. This cell differentiation is triggered by host-secreted peptides, called NCR that induce the disappearance of CtrA and FtsZ proteins by a yet unknown mechanism. Regarding the function of CtrA (inhibition of DNA replication and activation of cell division), the downregulation of this latter is consistent with the bacteroid phenotype. Thus, a plausible hypothesis is that CtrA may constitute a direct target of the NCR peptides. Hence, the physical interaction of NCR247 with multiple proteins such as FtsZ, GroEL, RpoH and ribosomal proteins, was already reported (Farkas *et al.*, 2014). Thus, it is reasonable to propose FcrX as a potential target of the NCR peptides that may interact physically with FcrX and stabilize the protein in order to downregulate the expression of CtrA and FtsZ proteins in the bacteroid. Moreover, when FcrX is overexpressed, the sensitivity of *S. meliloti* toward NCR247 increases as well. This result confirms that FcrX is somehow linked to NCR peptide effect. FcrX may be linked directly to the peptide as proposed above thus overexpressing the target of the NCR helps. To verify this hypothesis, we could realize an interaction test such as an affinity column, as it was realized in the study of (Farkas *et al.*, 2014) or a CoIP experiment on a strain of *S. meliloti* treated with a FLAG tagged NCR247 and by using antibodies directed against FcrX. Furthermore, it would be interesting to consider the NCR247 peptide as a potential therapeutic molecule. Indeed, as cited above this peptide is able to interact with intracellular targets, this feature can be exploited to disturb the bacterial physiology, eventually by targeting FcrX. In addition, the NCR247 peptide possesses antibacterial activity conferred by its cationic charge, which disturbs the negatively charged bacterial envelope by inducing perforation (Lima *et al.*, 2022).

The inoculation of legume plants *M. sativa* with a strain of *S. meliloti* that overexpress FcrX leads to bigger plants and a higher dry mass gain compared to *M. sativa* inoculated with a wild type strain. The overexpression of FcrX does not seem to act on the capacity of the bacteria to colonize the nodule neither on the level of differentiation. Thus, the hypothesis that the overexpression of FcrX affects the capacity of infection or increase the level of bacteroid differentiation are excluded. The conclusion we reached is that this overexpression may accelerate the downregulation of CtrA and FtsZ proteins, which could lead to an early formation of bacteroids and as a consequence the nitrogen fixation and the growth promotion. However,

these observations were made after 5 weeks post inoculation thus, to verify this hypothesis a kinetic monitoring should be realized on the plants inoculated with the overexpression strain and compared with plants inoculated with a wild type strain.

Furthermore, the overexpression of FcrX may be tested in other legume-rhizobium symbioses like *Glycine max* (soybean) interacting with *Bradyrhizobium japonicum* or *Sinorhizobium fredii*. Conveniently, both strains contain a *fcrX* copy in their genome. In addition, *G. max* does not express NCR peptides hence the rhizobia are not differentiated. Thus, it would be interesting to induce terminally differentiated bacteroids in this model. As explained in the introduction, the differentiation into bacteroid is advantageous for the symbiosis process and seems to be appeared later in the evolution. By overexpressing FcrX in a *Sinorhizobium fredii*, we could obtain the same growth gain in *G. max,* which can constitute a major breakthrough in agriculture since soybean constitutes one of most important crop worldwide and an important source of protein for food and feed (Pagano and Miransari, 2016).

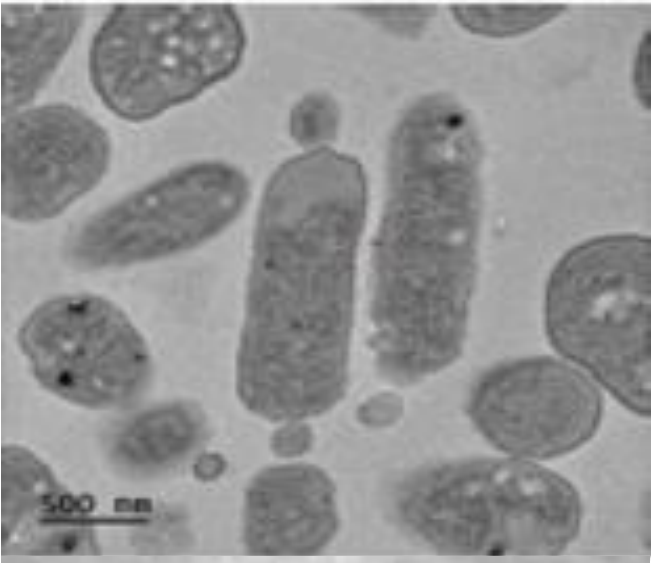
Conclusion

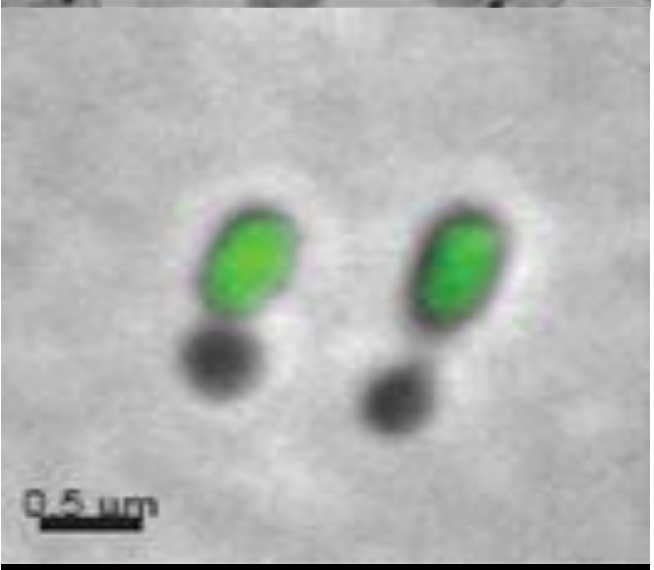
The study of the cell cycle regulation circuit in bacterial models is of the utmost importance. Indeed, the understanding of the mechanisms that govern this biological process, allows us to apprehend with greater ease the cell cycle in more evolved models, which are more complicated to study in laboratories. In addition, the cell cycle constitutes an interesting target for therapeutic molecules development. The discovery of the new cell cycle regulator FcrX in *S. meliloti* is one-step further in the unveiling of the cell cycle functioning of this bacterial model. This finding stresses the necessity to deepen our knowledge of the cell cycle regulation in non-conventional models. Moreover, the link of FcrX to the cell cycle and cell division makes it a potential target for therapeutic molecules design.

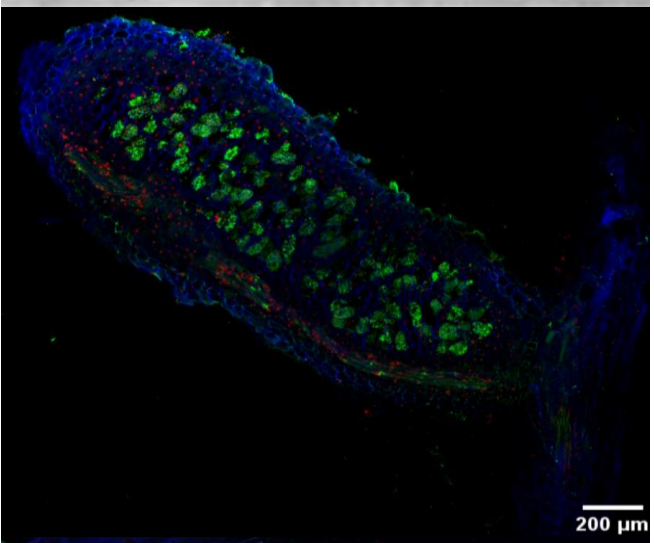
As mentioned in the introduction of this manuscript, the food demand is in constant increase; still the nitrogen is a limiting factor for the plant growth. To palliate to this latter, fertilizers are intensively used. However, the process of their production together with the consequences of their leaching are detrimental for the environment and are responsible of health issues. Thus, regarding the negative impact of fertilizers, the finding of novel alternatives is more necessary than ever. One of the solutions proposed is the use of rhizobia in order to promote symbiotic nitrogen fixation and increase the nitrogen enrichment of soils.

The understanding of the *S. meliloti* cell cycle, which is tightly linked to the symbiosis process with the host plant *M. sativa*, is one way towards the promotion of the biological nitrogen fixation for a safer increase of agricultural yields. This thesis project is positioned in this light. The positive effect of FcrX overexpression towards the plant growth during the symbiosis proved once again the critical role of the bacterial cell cycle regulation in the symbiosis process. Thus, this work reinforces the possibility of using the rhizobial cell cycle as a tool in order to increase the efficiency of symbiosis, at least in the model *S. meliloti* - *M. sativa*. However, this finding can be translated to other models regarding the conservation of FcrX in other rhizobial species.

Indeed, additional experiments in order to decipher in details the FcrX mode of action is primordial, notably, by unveiling the link between this latter and the degradosome complex as well as the potential involvement of the NCR peptides in FcrX stabilization during the symbiotic process.







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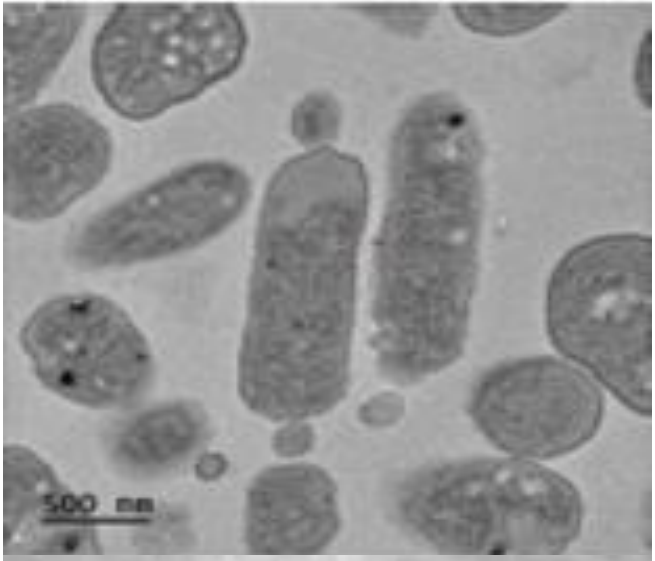

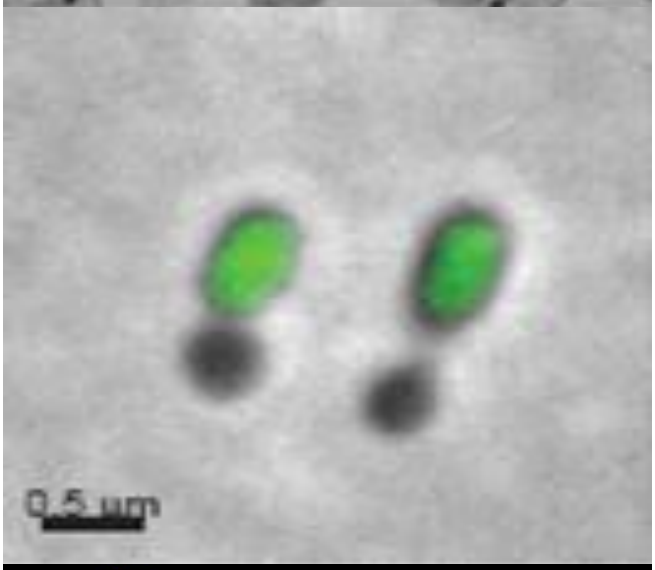

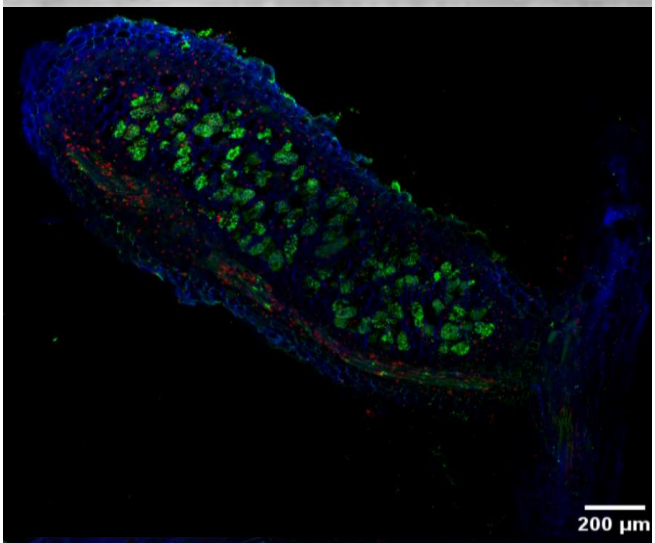

ANNEXES

Table S5 Strains used in second part of results

Description	Antibiotics	Source	Name (EB)
Sinorhizobium meliloti 1021	Sm (200µg/ml)	Biondi lab	**446**
Sinorhizobium meliloti *1021* ΔctrA::tet + pSRK-Km-*ctrA*	Sm (200µg/ml) + km (200µg/ml) + tc (10µg/ml) + 1mM IPTG	Pini et al, 2015	**249**
Sinorhizobium meliloti *1021* ΔfcrX::tet + pSRK-Gm-*fcrX*	Sm (200µg/ml) + Gm (20µg/ml) + tc (10µg/ml) + 100µM IPTG	This study	**1889**
Escherichia coli βHT101 pUT18 fcrX pKT25 clpP3	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3396**
Escherichia coli βHT101 pUT18 fcrX pKNT25 clpP3	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3397**
Escherichia coli βHT101 pUT18C fcrX pKT25 clpP3	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3398**
Escherichia coli βHT101 pUT18C fcrX pKNT25 clpP3	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3399**
Escherichia coli βHT101 pUT18 clpP3 pKT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3400**
Escherichia coli βHT101 pUT18 clpP3 pKNT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3401**
Escherichia coli βHT101 pUT18C clpP3 pKT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**2659**
Escherichia coli βHT101 pUT18C clpP3 pKNT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**2658**
Escherichia coli βHT101 pUT18 fcrX pKT25 cpdR	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3344**
Escherichia coli βHT101 pUT18 fcrX pKNT25 cpdR	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3345**
Escherichia coli βHT101 pUT18C fcrX pKT25 cpdR	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3346**
Escherichia coli βHT101 pUT18C fcrX pKNT25 cpdR	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3347**
Escherichia coli βHT101 pKT25 fcrX pUT18 cpdR	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3348**
Escherichia coli βHT101 pKT25 fcrX pUT18C cpdR	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3349**
Escherichia coli βHT101 pKNT25 fcrX pUT18 cpdR	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3350**
Escherichia coli βHT101 pKNT25 fcrX pUT18C cpdR	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3351**
Escherichia coli βHT101 pUT18 fcrX pKT25 rcdA	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3022**
Escherichia coli βHT101 pUT18 fcrX pKNT25 rcdA	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3023**
Escherichia coli βHT101 pUT18C fcrX pKT25 rcdA	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3024**
Escherichia coli βHT101 pUT18C fcrX pKNT25 rcdA	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3025**
Escherichia coli βHT101 pUT18 rcdA pKT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3026**
Escherichia coli βHT101 pUT18 rcdA pKNT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3027**
Escherichia coli βHT101 pUT18C rcdA pKT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3028**
Escherichia coli βHT101 pUT18C rcdA pKNT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3029**
Escherichia coli βHT101 pUT18 fcrX pKT25 clpX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3030**
Escherichia coli βHT101 pUT18 fcrX pKNT25 clpX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3031**
Escherichia coli βHT101 pUT18C fcrX pKT25 clpX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3032**
Escherichia coli βHT101 pUT18C fcrX pKNT25 clpX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3033**
Escherichia coli βHT101 pUT18 clpX pKT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3034**
Escherichia coli βHT101 pUT18 clpX pKNT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3035**
Escherichia coli βHT101 pUT18C clpX pKT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3036**
Escherichia coli βHT101 pUT18C clpX pKNT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3037**
Escherichia coli βHT101 pUT18 fcrX pKT25 clpP2	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3311**
Escherichia coli βHT101 pUT18 fcrX pKNT25 clpP2	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3312**
Escherichia coli βHT101 pUT18C fcrX pKT25 clpP2	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3313**
Escherichia coli βHT101 pUT18C fcrX pKNT25 clpP2	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3314**
Escherichia coli βHT101 pUT18 clpP2 pKT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3315**
Escherichia coli βHT101 pUT18 clpP2 pKNT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3316**
Escherichia coli βHT101 pUT18C clpP2 pKT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3317**
Escherichia coli βHT101 pUT18C clpP2 pKNT25 fcrX	Amp (100µg/ml) + Km (50µg/ml) + Xgal (40µg/ml) + 500µM IPTG	This study	**3318**
Sinorhizobium meliloti 1021 pSRK Plac ClpP1-cfp	Sm (200µg/ml) + Gm (20µg/ml)	This study	**3076**
Sinorhizobium meliloti 1021 pSRK Plac ClpX-cfp	Sm (200µg/ml) + Gm (20µg/ml)	This study	**3110**
Sinorhizobium meliloti 1021 pSRK-Gm	Sm (200µg/ml) + Gm (20µg/ml)	This study	**350**
Sinorhizobium meliloti 1021 pSRK-Km	Sm (200µg/ml) + Km (100µg/ml)	This study	**261**

Table S6 Primers used in the second part of the results

Primer's name	Sequence (5'-3')
fcrX forward for BACTH	TCTAGAGATGAAAGCACGTGAGAGCCTAGT
fcrX reverse for BACTH	TACCCGGGGGGCGCGCAGCCGCGCACCCG
ClpP1 forward for BACTH	GGTCTAGAGATGGCCATCAACTTGCAGAAGC
ClpP1 reverse for BACTH	TACCCGGGGGCTCGACTGGCGGCGCAGG
ClpP2 forward for BACTH	TCTAGAGATGACGGAATACAAGAAGCCG
ClpP2 reverse for BACTH	TACCCGGGGCTGCAGCGTACGCGAAGGAAC
ClpP3 forward for BACTH	TCTAGAGATGCGGGTTCTACTGATCG
ClpP3 reverse for BACTH	GGTACCCGGGCGGTTTCCAGGTAGTCG
RcdA forward for BACTH	TCTAGAGATGCGGGTTCTACTGATCG
rcdA reverse for BACTH	GGTACCCGGGCGGTTTCCAGGTAGTCG
CpdR1 forward for BACTH	TCTAGAGATGCGGGTTCTACTGATCG
CpdR1 reverse for BACTH	GGTACCCGGGCGGTTTCCAGGTAGTCG
ClpX forward for BACTH	TCTAGAGATGCGGGTTCTACTGATCG
ClpX reverse for BACTH	GGTACCCGGGCGGTTTCCAGGTAGTCG
yfp/CFP reverse	GGGGATCCTTACTTGTACAGCTCGTCCATGCCG
ClpP-NdeI-fw	GGCATATGAGAAATCCAGTTGATACG
ClpP-BAmHIrev	GGGGATCCTTCCATGCCCTCGACCGCATC
ClpP2-NdeI-f	GGCATATGCGGAACGACGACGACC
ClpP2-BamHI-rev	GGGGATCCTCAAATCTCCGCGATGGAGGTG
ClpP3-SacI-fw	GGGAGCTCATCTATTCCCGCCTGCTGCG
ClpP3-BamHI-rev	GGGGATCCTCATGCATCACGTTCAGCGCC
ClpX-NdeI-fw	GGCATATGAGCAAGGTCAGCGGTAGC
ClpX-BamHI-rev	GGGGATCCTCAAGCCGAAACGTTGGTCTTC