



Anaerobic respiration diversification in *Agrobacterium fabrum* C58

Solène Lecomte

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Solène Lecomte. Anaerobic respiration diversification in *Agrobacterium fabrum* C58. Symbiosis. Université de Lyon, 2019. English. NNT : 2019LYSE1231 . tel-03013488

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N°d'ordre NNT : xxx



THESE de DOCTORAT DE L'UNIVERSITE DE LYON

opérée au sein de
l'Université Claude Bernard Lyon 1

École Doctorale N° 351
Évolution Écosystème Microbiologie Modélisation

Spécialité de doctorat : Microorganismes, interactions, infections

Discipline : Biologie

Soutenue publiquement le 18/11/2019, par :

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Anaerobic respiration diversification in *Agrobacterium fabrum* C58

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Résumé

La rhizosphère, zone sous l'influence des racines de plantes, est un environnement riche en microorganismes dû à l'exsudation de 16 à 50% de composés carbonés issus de la photosynthèse. Ces derniers sont responsables de l'attraction des bactéries à la surface des racines qu'elles colonisent avec succès lorsqu'elles possèdent des traits spécifiques nommés « compétence rhizosphérique ». La communauté microbienne rhizosphérique est influencée par des facteurs environnementaux changeant tels que le pH, la qualité et la quantité des exsudats racinaires et le flux en O₂. En effet, les jeunes racines modifient à leur contact la pression partielle de l'oxygène présent dans le sol. Cette variation dans la concentration en O₂ contraint les bactéries à adapter leur physiologie et à diversifier leur métabolisme en utilisant par exemple des accepteurs terminaux d'électrons (TEA) alternatif à l'oxygène pour la respiration anaérobie. La respiration anaérobie la plus étudiée dans la rhizosphère est la dénitrification qui correspond à la réduction successive du nitrate (NO₃⁻) en diazote (N₂) via la production de nitrite (NO₂⁻), d'oxyde nitrique (NO), et d'oxyde nitreux (N₂O).

Le genre bactérien *Agrobacterium* vit dans différents habitats (sol, rhizosphère, plante hôte), et doit ainsi faire face à différentes contraintes environnementales, tels que la disponibilité en substrats carbonés et azotés, la limitation en O₂ ... Ce genre est organisé en population et plusieurs espèces génomiques sont communément retrouvés dans les mêmes niches écologiques. Selon le principe d'exclusion compétitive, les espèces peuvent coexister uniquement si elles sont adaptées à des niches écologiques différentes. De plus, les agrobactéries qui possèdent le plasmide Ti portant les gènes de virulence sont capables de modifier génétiquement leur hôte afin de construire une niche spécifique, la tumeur végétale. Pour coloniser la rhizosphère et la tumeur, *Agrobacterium* a développé la capacité à exploiter des ressources diverses telles que les opines et probablement la capacité à respirer dans des conditions micro-aérophiles largement retrouvées dans ces environnements. La capacité à dénitrifier, seule respiration anaérobie validée chez les agrobactéries, n'est pas partagée par toutes les espèces d'*Agrobacterium*. Parmi elles, *A. fabrum* C58 est partiellement dénitrifiante car dépourvue de l'enzyme catalysant la réduction du N₂O.

L'hypothèse de cette thèse était que la diversification de la respiration anaérobie est une explication fortement probable de la coexistence des agrobactéries et de leur distribution dans des niches spécifiques de la rhizosphère. Dans le but de tester cette hypothèse, nous devons d'abord mieux caractériser la voie de dénitrification puis révéler d'autres respirations anaérobies pouvant se mettre en place dans la rhizosphère. Pour cela, nous avons utilisé la souche modèle *A. fabrum* C58. Nous avons tout d'abord caractérisé la première étape de dénitrification chez *A. fabrum* C58 et son rôle dans la survie et la colonisation en combinant la construction de mutants et l'évaluation de la valeur adaptative. Dans le but d'identifier de nouveaux régulateurs impliqués dans la dénitrification, nous

avons construit une banque de mutants par insertion aléatoire d'un transposon chez *A. fabrum* C58. L'utilisation de cette banque en conditions de dénitrification a permis de révéler les gènes impliqués dans sa régulation. Puis, nous avons mis au point « TEAMS, for TEA Method Screening » une méthode de criblage d'accepteurs terminaux d'électrons en anaérobiose. Ainsi, la mise en place de respirations anaérobies en présence de métabolites exsudés par la plante a été évaluée. Ainsi, nous avons démontré chez *A. fabrum* C58 que (i) NapA est une réductase impliquée dans la dénitrification et dans la survie mais non pas dans la colonisation racinaire, (ii) NopR est un ARN non codant impliqué dans le contrôle de la dénitrification, et (iii) *A. fabrum* C58 est capable de respirer en anaérobiose 7 composés exsudés par les racines de maïs. Pour répondre entièrement à notre hypothèse de départ, les différentes approches mises en œuvre chez *A. fabrum* C58 devront être appliquées chez d'autres espèces d'*A. tumefaciens*.

Abstract

The rhizosphere, the soil volume affected by plant roots, is an important microbial hotspot through the exudations of root metabolites representing 16–50% of plant photosynthetically fixed carbon. As a result of this carbon flux, bacteria are attracted to the surface of the roots that they successfully colonize if they possess specific genetic and physiologic traits named “rhizosphere competence”. The rhizosphere microbial community is shaped by changing environmental factors that occur in rhizosphere, such as pH, quantity and quality of root exudates and oxygen flux. Indeed, the roots of growing plants modify the soil oxygen partial pressure in their immediate vicinity. This variation in oxygen concentration constrains bacteria to adapt their physiology and to develop flexible metabolic pathways, such as the use of alternative terminal electron acceptors (TEAs) for anaerobic respiration. The most studied anaerobic respiration in the rhizosphere is denitrification. During denitrification, a variety of intermediate compounds are produced as nitrate is reduced stepwise to N_2 , including the nitrite (NO_2^-), and the gases nitric oxide (NO) and nitrous oxide (N_2O).

Agrobacterium genus lives in different habitats (soil, rhizosphere, host plants), and hence faces different environmental constraints, such as C and N substrate availability, oxygen limitation etc. *Agrobacterium* is organized in populations and several strains and genomic species are commonly found in the same ecological niches. Because complete competitors cannot coexist, co-occurring species must be adapted to partly different ecological niches. This co-occurrence of *Agrobacterium* strains may be allowed by species-specific genes. In addition, *Agrobacterium* harboring virulence genes carried by the Ti plasmid are able to modify the genome of their host and to construct a specific niche,

i.e. the plant tumor. To colonize the rhizosphere and tumor environments, *Agrobacterium* have evolved the capacity to exploit diverse resources, such as opines, and probably to respire under micro-oxic condition largely encountered in these niches. In agrobacteria, denitrification is the only one validated anaerobic respiration. The ability to denitrify is not found in all species of *Agrobacterium* may explaining partially their distribution in different habitats. Among *Agrobacterium* genus, *A. fabrum* C58 is a partial denitrifier that lacks the nitrous oxide reductase.

The hypothesis of this thesis was that anaerobic respiration diversification, notably denitrification, could at least partly explain the coexistence of Agrobacteria and their distribution in specific niches in the rhizosphere. In order to test this hypothesis, we need first to better characterize denitrification respiration and to evidence whether other anaerobic respirations may occur in the rhizosphere of plants. To do so, we used the model strain *A. fabrum* C58. First, we characterized the first step of denitrification and its role in the fitness and root colonization by constructing mutant strain and performing competition assays. We constructed a transposon mutant library in *A. fabrum* C58 and used it to reveal key genes involved in denitrification pathway. We also set up a new method named “TEAMS” for TEAs Method Screening to screen putative TEAs under anoxic condition. Primary metabolites exuded by maize roots were screened for their potential use as TEAs. We thus demonstrated in *A. fabrum* C58 that (i) NapA is a nitrate reductase involved in denitrification and fitness but not in root colonization, (ii) NopR is a small RNA involved in denitrification control and for the first time (iii) *A. fabrum* C58 is able to anaerobically respire 7 compounds known to be exuded by maize roots. To entirely validate our hypothesis, other *Agrobacterium tumefaciens* strains need to be tested. Approaches (TEAMS and Tn-seq) used on *A. fabrum* C58 have now to be applied to these strains.

Remerciements

Je tiens à commencer ces remerciements par Sara Hallin, Denis Faure et Laurent Philippot qui ont accepté d'évaluer mes travaux de thèse. Et également Céline Brochier qui a accepté de compléter ce jury au pied levé. J'espère que ce manuscrit et la soutenance à suivre vous plairont et enrichiront vos connaissances.

Bien sur ces remerciements continuent avec ma chef Zahar. Ça fait maintenant 5 ans qu'on vit l'histoire « recherche » ensemble. On a traversé beaucoup de choses, des épreuves, des moments de joie, de partage. On s'est toujours battu côte à côte et progressé ensemble. Je sors grandie de cette thèse grâce à toi et dotée d'une force immense. Et je sais que je mènerai toujours à bien mes projets quels qu'ils soient grâce à ce que tu m'as transmis. Juste merci. Dédicace spéciale à tes filles que j'ai apprises à apprivoiser et que j'apprécie beaucoup. Je remercie également Xavier pour les discussions enrichissantes. Profite bien de ta retraite, de ta petite fille et des nombreux autres à venir !

Je tiens à remercier également les membres de mon comité de pilotage : Phil et Wafa. Merci Phil de m'avoir ouvert les portes de ton labo et de m'avoir conseillé. Merci Wafa pour tes précieux conseils et ton prêt de matériel.

Je remercie également toutes les personnes du MAP avec qui j'ai eu la chance de travailler. Erwan, le savant fou des mutants, merci pour tous tes conseils et ton aide précieuse. Florence H, ma chef de M2, tu as continué à être là pour moi, à échanger et t'investir, merci. Et les autres que j'ai côtoyés lorsque je me cachais là-bas ;)

Grosse pensée pour Corinne et Audrey qui font tourner le labo, à Danis qui a toujours su répondre à mes demandes de dernières minutes, à Élise à qui je laisse mes maïs pourrir pendant 2 mois à la serre, et merci à tous ceux que j'ai sollicités lorsque j'avais des questions existentielles (souvent à 8h le lundi matin ou à 17h30 le vendredi soir ...)

Merci à Agro de ne pas t'être laissée faire et de m'avoir appris la persévérance et la patience (ou presque). Merci à Word, le pire logiciel au monde pour écrire une thèse !

Maintenant venons-en à mes acolytes !

Mes copines. Béa, pour commencer parce que je sais que tu vas être jalouse sinon :p Ma petite esclave du tiers monde qui m'a fait des manip juste pour m'aider parce que c'est fait pour ça les copines. On a vécu pas mal de choses ensemble entre le sport au labo dans la salle radioac', le tatouage, nos folles chevauchées en trot même si une fois j'ai pris le métro ... Et c'est pas fini avec ton mariage où j'aurai l'honneur (vraiment) d'être ta demoiselle d'honneur. Elo, presque deux ans maintenant qu'on partage des discussions sans fin, des préparations aux soirées / mariages, une passion commune pour les

vêtements de sport et pour le vin aussi. Lolo, tu me fais rêver avec ton accent et des expressions (tu files un mauvais coton !! Ah ouais ?!), toujours là pour apporter ta bonne humeur et ta nonchalance et ça fait bien, pour nous calmer un peu ^^ . Marin, j'aimerais parfois revenir en arrière pour revivre nos pauses café infinies et nos discussions scientifique (parfois) ou pas (souvent) puis je pense que depuis que tu es partie à Sainté, j'ai la chance de voir ton doudou de l'horreur une fois de temps en temps et ça, ça fait ma journée. On va vraiment bien se marrer à Lisbonne toutes les 5.

Mon besto Guillaume. Tu es loin et je ne t'oublie pas. Nos conversations autour d'un café ou pas me manquent, nos échanges scientifiques aussi (j'aimais bien surtout quand tu faisais mes stats). On s'est un peu loupé cette année, mais promis on fera mieux l'année prochaine ! Profite bien de l'expérience post doc chilien je viendrais peut être te voir (si je meurs pas dans l'avion) !

Les potos maintenant. Jonath, je sais qu'on a pas le droit d'être pote parce qu'on « mélange pas vie pro et vie perso » mais maintenant que j'ai fini c'est bon ? ^^ Merci pour ce temps passé sur la plateforme à parler de tout et de rien, merci pour tes conseils de vie, j'essayerai peut-être de les suivre. Juju, 4 ans maintenant qu'on partage de bons moments et pour que ça dure promis on jouera plus jamais à Photosynthesis :p Flo V, si tu continues à faire du pâté et de la tapenade on sera amis pour la vie, la balle est dans ton camp ! Thibault, ces 2 premières années de thèse dans ton bureau ont été complètement folles, je me souviendrais toujours de ta blague avec l'alarme incendie, à quand une virée en Belgique ? Et enfin Kévin, j'aime bien de te laisser croire que tu m'apprends la science, surtout autour d'une bière !

La team Z, la famille : Wiwi et notre façon de se dire bonjour, de craquer dans le bureau en écoutant Rihanna ou Beyoncé, ta façon de jouer au billard et de me moquer gentiment surtout à Lisbonne. Et Momo, ma coloc d'un temps, on a pas eu beaucoup l'occam de se voir pendant ces 3 ans mais ça nous empêche pas d'être connectée et de soutenir à 4j d'intervalle sans concertation ... Les grands esprits se rencontrent :) Théo, à toi de prendre la relève et te sortir Zahar de son chagrin de nous perdre cette année ! Fabiola, merci pour ta bonne humeur et tes chocolats lors de la dernière ligne droite :)

Et bien sûr Dudu, Cocotte, Mel, Maxime (du MAP). Samsam le brave dans ce monde de brutes, merci pour tes présentations auxquelles on comprenait rien mais qui nous faisaient bien marrer ! Laurène je ne sais pas si je dois te remercier pour tes discussions à sens unique mais en tout cas je te remercie pour tes talents de dessinatrices. Lucas merci pour tes anecdotes trop longues et pour m'avoir aidé lorsque mon cerveau refusait d'apprendre à se servir d'un nouveau logiciel. Merci à Carole de prendre des nouvelles régulièrement, tkt tout va bien ;) Merci aussi d'avoir tout fait pour assister à ma soutenance, d'avoir gardé Fauvette plus d'une fois et d'être mon repère breton parmi ces lyonnais ^^ Zazou et Laura Camus avec qui j'ai passé des soirées filles au top. David, j'espère que je resterai ta conseillère photo même si je suis plus au labo.

Mes stagiaires. Je suis obligée de commencer par Grégory, mon chouchou. Tu étais le premier, le crash test et je pense que ça s'est bien passé puisque tu commences ta thèse cette année, bonne chance à toi ! Merci aux filles, Amel et Manon, je n'ai pas eu l'occasion de vous accorder autant de temps que j'aurais aimé. Bon courage pour votre M2 !!

Petite ode aux doctorants. Les vieux qui ne sont plus que des noms ou des anecdotes (Seb, Jordan, Mag), les anciens que j'ai vus passer (Antoine, Ben, Quentin), ceux qui ont eu la force d'arrêter avant qu'il ne soit trop tard, les actuels et les nouveaux qui ont encore tout à faire. Même si la recherche ne veut pas toujours de nous, il faut garder à l'esprit que faire une thèse c'est une expérience de vie dont on sort toujours plus grand, plus fort. La recherche n'est pas une fatalité, regardez Quentin, Mag, Juju et Zazou =) Mais acquérir toutes les compétences transversales possibles pendant son doctorat, c'est la vraie bonne astuce pour s'en sortir. Alors haut les cœurs !

A Fauvette, ma petite boule de poils et d'amour, pour nos siestes de « 20min » pendant la rédaction dans les bras l'une de l'autre.

Ma famille qui ne cesse de s'agrandir. Ma maman qui pense toujours à moi et qui me soutient toujours. Mon papa qui je le sais est fier de sa fille. Mon frère, ma sœur, ma Tata Lise, mes cousins et tous les enfants. Merci d'avoir été là, d'avoir écouté mes doutes existentiels et de m'avoir rassuré et bon courage pour survivre à la soutenance ! Merci aussi à ma belle-famille pour leur accueil chaleureux.

Et enfin, merci à Jordan, on a commencé cette histoire ensemble, on l'a vécu ensemble, on la termine ensemble et ça c'est beau <3 C'est notre première grande aventure à deux (sans compter nos nombreuses vacances ensemble), et ce ne sera pas la dernière ! On me souffle dans l'oreillette qu'un tour du monde nous tend les bras voire l'ouverture d'un bar à cocktails ? =D

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Introduction

Preamble: general context on microbial diversity and their anaerobic respiration strategies

1 Metabolism diversity and flexibility: role in microbial colonization

Over three decades of molecular-phylogenetic studies, researchers have compiled an increasingly robust map of evolutionary diversification showing that the main diversity of life is microbial, distributed among three primary relatedness groups or domains: Archaea, Bacteria, and Eukarya. The general properties of representatives of the three domains indicate that the earliest life was based on inorganic nutrition and that photosynthesis and use of organic compounds for carbon and energy metabolism came comparatively later (Pace, 1997). The application of molecular methods to study natural microbial ecosystems has resulted in the **discovery of many unexpected metabolic pathways**. These microbial metabolic pathways play key roles in maintaining multiple ecosystem functions and services simultaneously, including nutrient cycling, primary production, litter decomposition and climate regulation (van der Heijden et al., 2008; Wagg et al., 2014; Bardgett et al., 2014). Microbial communities colonized different environments which can be divided in (i) global and heterogeneous environments such as water, soil, atmosphere; (ii) host environments such as human, plants, insects; (iii) agronomic environments such as cheese, yogurt, beer and wine and (iv) extreme environments such as deep-sea hydrothermal vents, ice, acidic or alkaline lakes, desert, mines. The ability of **microorganisms to colonize a wide range of environments is probably due to their metabolism diversity and flexibility**. For example, in frozen water ecosystems, microbes are spatially organized according to the different strategies used to fulfil their energy requirement (Boetius et al., 2015) (**Figure 1**). Frozen water ecosystems are divided in snow, supraglacial and subglacial habitats and each one accommodates specific species. Snow and supraglacial ice are dominated by photosynthetic microorganisms, including cyanobacteria, ice algae and snow algae. The ice surface is colonized by heterotrophic groups, such as members of the *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* due to the deposition of carbon and nutrients that creates favorable growth conditions (Musilova et al., 2015). The subglacial habitats are dominated by chemolithoautotrophic communities. Microbial communities able to metabolize metals or nitrogen oxides can colonize the subglacial outflow. In the sediment, these microorganisms use the minerals and carbon substrates to generate energy through redox reactions.

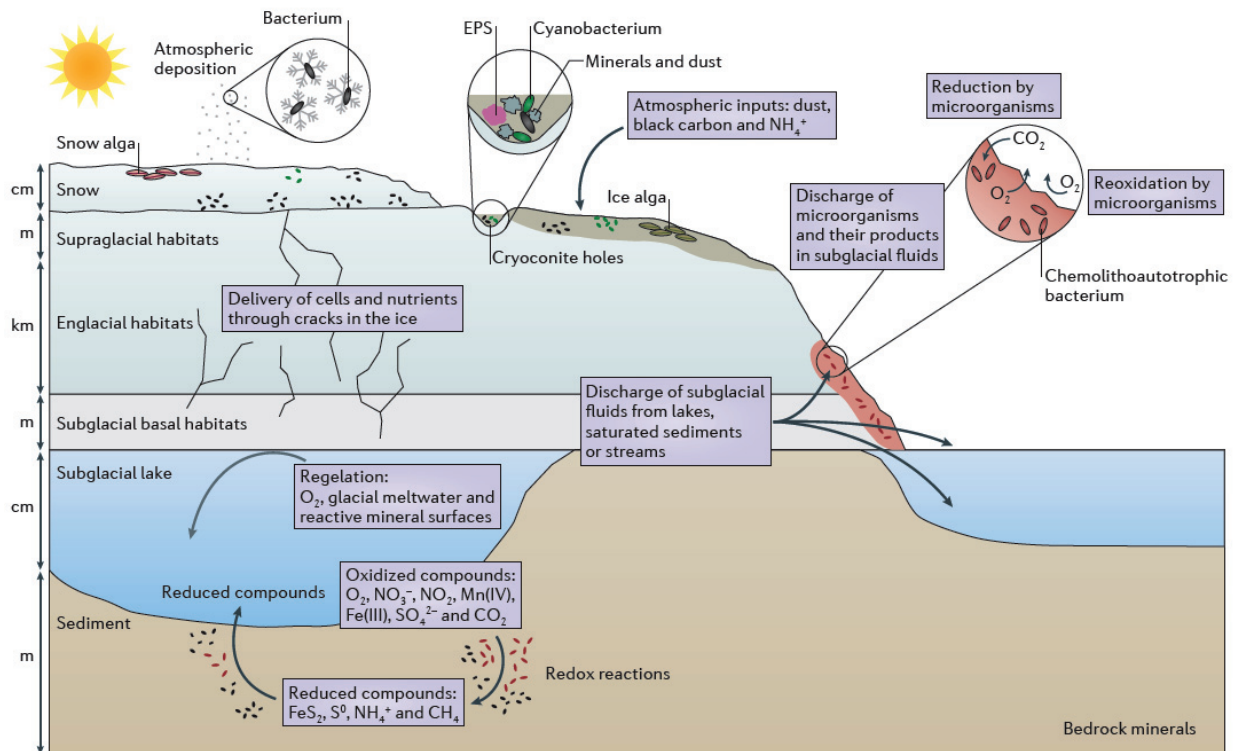


Figure 10: Biogeochemical processes in snow, supraglacial and subglacial ecosystems. Example of microbial colonization of different frozen habitats through different strategies used to fulfil their energy requirement. Habitats are on the left. Microorganisms are illustrated by colored bacilli. Strategies are described in violet rectangles. From Boetius *et al.* (2015).

Microbial diversification of metabolic pathways such as those involved in respiration could be a evolutive strategie of microorganisms to colonize wide range of environment.

2 Microbial respiration diversity

Respiration can be divided in two categories: aerobic respiration when oxygen is present and anaerobic respiration when oxygen is absent. Microbial anaerobic respiration is the most diverse on Earth. Indeed, a high diversity of compounds can be used as terminal electrons acceptors (TEAs), such as metals, radionuclides, nitrogen compounds or carbon compounds (Richardson, 2000; Lecomte *et al.*, 2018). Anaerobic respirations have been well described in water environments. *Geobacter* and *Shewanella oneidensis*, model strains to study anaerobic respiration, are able to use at least Fe(III), Mn(IV), nitrate, fumarate, arsenic oxyanions and U(VI) as TEAs (Lovley *et al.*, 1991; Beliaev *et al.*, 2005; Klonowska *et al.*, 2005).

Another interesting environment for studying anaerobic respiration is the rhizosphere, a densely populated soil area influenced by root activity (Hiltner, 1904; Haichar *et al.*, 2014) where the oxygen fluctuates between rapid consumption and poor production (Kuzakov & Razavi, 2019). In addition,

diverse compounds are found in the rhizosphere, such as Fe, Mn and NO_3^- which could serve as TEAs for rhizospheric microbial communities (**Figure 2**).

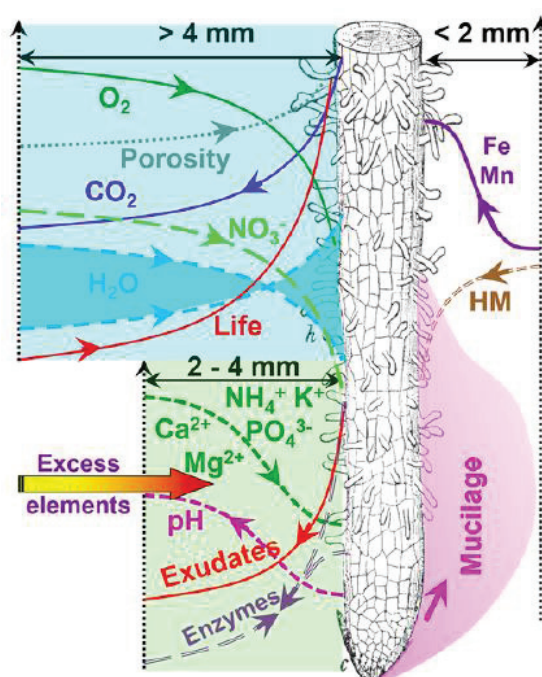


Figure 11. Distribution of the most investigated parameters in the rhizosphere: gases, root exudates, nutrients, elements heavy metals (HM), pH, enzymes activities and microorganisms (Life). The rhizosphere is a heterogenous environment where pH, soil structure, water content, oxygen flux, C nutrient availability, and microbial activities fluctuate. The complexity of the rhizosphere is explained by the distribution of these various parameters across and along the roots but also by the continuum of these parameters between root surface and bulk soil and finally by the temporal changes of these parameters with root growth. From Kuzyakov and Razavi, (2019).

Interestingly, plant roots secrete a wide range of compounds into the rhizosphere, with around 21% of total photosynthetically fixed carbon being transferred into the rhizosphere through root exudates (Whipps, 1990; Nguyen, 2003; Haichar et al. , 2014). This exudation participates in the complex organization of rhizospheric microbial life. Indeed, in maize rhizosphere and non-rhizosphere compartments, microbial populations are structured according to their metabolic abilities (Zhalnina et al., 2018). Remarkably, carbohydrates, amino acids and amides are discriminating substrates between bulk soil and rhizoplane microbes (Baudoin et al., 2001) (**Figure 3**). **These exuded substrates could also shape microbial communities through their use as TEAs.** However, little is known about the potential use of root exudates as TEAs for microbial anaerobic respirations.

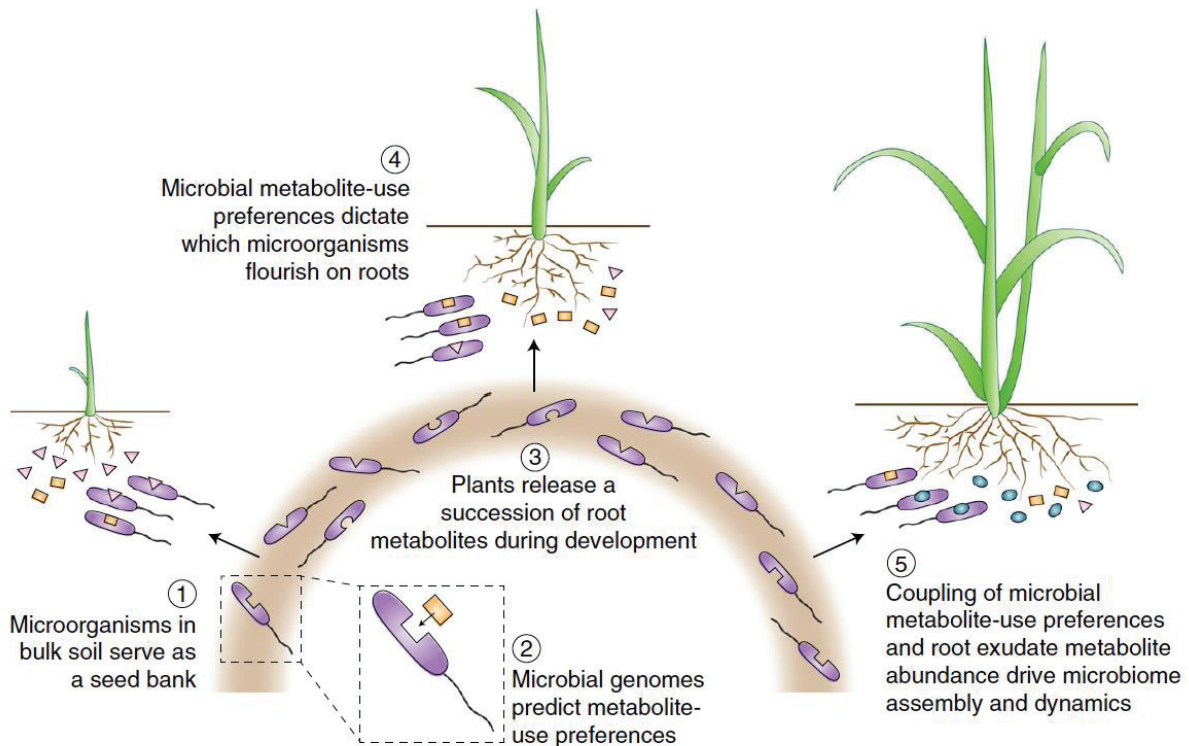


Figure 12: Microbial substrate utilization traits as a major driver of rhizosphere microbiome assembly. The substrate-use abilities of bulk soil microorganisms will determine which microorganisms will colonize the roots and flourish there. The type of released exudates will evolve with plant development leading to the evolution of the microbial community. Bacteria are shown in violet. Roots exudates are illustrated by different shapes diffusing from plant roots. From Beattie (2018).

The best studied anaerobic respiration in the rhizosphere is denitrification (for review, see Chapuis-Lardy et al., 2007; Hu et al., 2015). It uses nitrate as a TEA and yields energy to sustain bacterial growth. It includes nitrate, nitrite, nitric oxide and nitrous oxide reductions by different reductases (Zumft, 1997). Genes encoded for denitrifying reductases are well known, however, except for some regulators and two-component systems (Baek et al., 2008), little is known about denitrification regulation *in vitro* and no data is available for denitrification regulation *in planta*.

Bacteria successfully colonize the rhizosphere when they possess specific genetic and physiologic traits named “**rhizosphere competence**”. The denitrification process has been accepted as a rhizosphere competence trait (Ghirardi et al., 2012). Indeed, denitrification genes (nitrate and nitrite reductase) have been demonstrated to confer a competitive advantage in rhizosphere colonization for *Pseudomonas fluorescens* YT101 (Philippot et al., 1995; Ghiglione et al., 2000; Ghiglione et al., 2002). **The diversification of anaerobic respiration can be also considered as an essential trait for rhizosphere competence.** Further studies are needed to deeply understand anaerobic respiration diversity and flexibility and its role in rhizosphere colonization. To reach this exciting research

challenge, the use of rhizospheric model strains and a combination of a wide range of scientific expertise are required.

Among well-known rhizospheric model strains, *Agrobacterium* is a very interesting genus colonizing rhizosphere and tumor niches (**Figure 4**), through the presence of its Ti plasmid and metabolic versatility (Dessaix & Faure, 2018). Rhizosphere and tumor environments are characterized by variation in oxygen concentration or low oxygen content, respectively (Lecomte et al., 2018; Deeken et al., 2006). Interestingly, *Agrobacterium fabrum* C58 is a partial denitrifier (Baek & Shapleigh, 2005) known to colonize efficiently rhizosphere and tumor niches (Moore et al., 1997; Chèneby et al., 2004). Thus, *A. fabrum* C58 is an interesting strain to study more deeply denitrification regulation and to evidence new anaerobic respiration pathways and their involvement in root and tumor colonization.

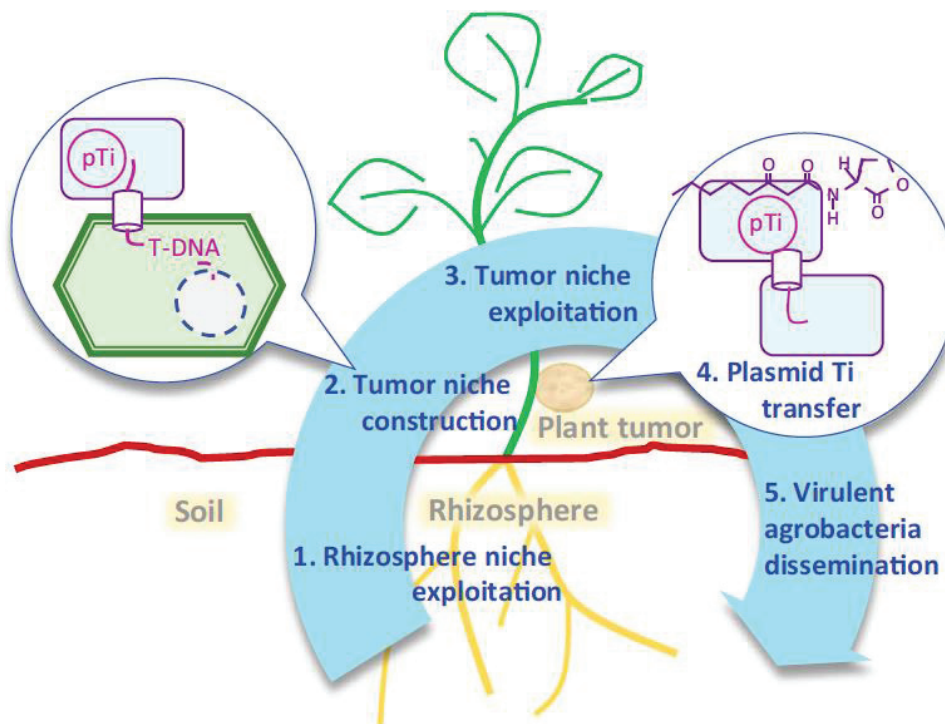


Figure 13. Ecological niches of *Agrobacterium*. Saprophytic and pathogenic *Agrobacterium* populations efficiently colonize the rhizosphere of host and non-host plants. Plant tumor is a novel ecological niche constructed by virulent *Agrobacterium* through the transfer of T-DNA in the plant host genome. Opine, a tumor resource, is exploited by *Agrobacterium* pathogens conferring a selective advantage. The Ti plasmid conjugative transfer is promoted by opines contributing to the maintenance and propagation of the virulence genes. From Dessaix and Faure, (2018).

3 Structure of the manuscript

This manuscript will be organized in 5 parts. The first part consists in literature review with the objective of compiling available information on the possible diversification and study of anaerobic respirations in the rhizosphere. Thus, this part is divided into (i) the diversification of anaerobic respiration strategies in the rhizosphere (**published review in *Frontiers in Environmental Science, soil processes***), (ii) a focus on denitrification pathways in the rhizosphere, and (iii) a presentation of *A. fabrum* C58 as a model strain used in this thesis.

The second part deals with the involvement of NapA in the fitness and rhizosphere colonization. To do so, a *napA* mutant of *A. fabrum* C58 was constructed, its growth and root colonization capacity were then evaluated under anoxic conditions *in vitro* and *in planta*. In addition, the role of small RNA NopR in denitrification regulation was evidenced.

The purpose of the third part was to evidence regulators involved in denitrification control *in vitro* and *in planta*. To do so, a transposon mutant library was constructed in *A. fabrum* C58 and essential genes were uncovered after library sequencing (Tn-seq). This library was then inoculated under anoxic conditions *in vitro* and on maize roots in a greenhouse. Library sequencing is in progress.

The fourth part was to evidence whether other anaerobic respirations than denitrification may occur in plant rhizosphere and more precisely if primary metabolite root exudates could serve as TEAs. To do so, the composition of maize root exudates was determined. Then, a method for anaerobic respiration screening of carbon and nitrogen sources was set-up. The growth of *A. fabrum* C58 was evaluated on different carbon and nitrogen sources exuded by maize plant.

Finally, the fifth part consists of a general discussion of the whole results and some perspectives on which this thesis work could lead.

Literature review

Literature review

1 Anaerobic respiration in the rhizosphere

In this part, anaerobic respirations that may occur in the rhizosphere has been reviewed followed by a focus on the respiration of nitrogen oxide, known as denitrification.

1.1 Diversifying anaerobic respiration strategies to compete in the rhizosphere

This part was published in *Frontiers in Environmental Sciences*, microbe-mediated processes in soils.



Diversifying Anaerobic Respiration Strategies to Compete in the Rhizosphere

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OPEN ACCESS

Edited by:

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Federal University of Pampa, Brazil

Reviewed by:

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Specialty section:

This article was submitted to
Soil Processes,
a section of the journal
Frontiers in Environmental Science

Received: 27 September 2018

Accepted: 01 November 2018

Published: 23 November 2018

Citation:

Lecomte SM, Achouak W, Abrouk D,
Heulin T, Nesme X and Haichar FZ
(2018) Diversifying Anaerobic
Respiration Strategies to Compete in
the Rhizosphere.
Front. Environ. Sci. 6:139.
doi: 10.3389/fenvs.2018.00139

The rhizosphere is the interface between plant roots and soil where intense, varied interactions between plants and microbes influence plants' health and growth through their influence on biochemical cycles, such as the carbon, nitrogen, and iron cycles. The rhizosphere is also a changing environment where oxygen can be rapidly limited and anaerobic zones can be established. Microorganisms successfully colonize the rhizosphere when they possess specific traits referred to as rhizosphere competence. Anaerobic respiration flexibility contributes to the rhizosphere competence of microbes. Indeed, a wide range of compounds that are available in the rhizosphere can serve as alternative terminal electron acceptors during anaerobic respiration such as nitrates, iron, carbon compounds, sulfur, metalloids, and radionuclides. In the presence of multiple terminal electron acceptors in a complex environment such as the rhizosphere and in the absence of O₂, microorganisms will first use the most energetic option to sustain growth. Anaerobic respiration has been deeply studied, and the genes involved in anaerobic respiration have been identified. However, aqueous environment and paddy soils are the most studied environments for anaerobic respiration, even if we provide evidence in this review that anaerobic respiration also occurs in the plant rhizosphere. Indeed, we provide evidence by performing a BLAST analysis on metatranscriptomic data that genes involved in iron, sulfur, arsenate and selenate anaerobic respiration are expressed in the rhizosphere, underscoring that the rhizosphere environment is suitable for the establishment of anaerobic respiration. We thus focus this review on current research concerning the different types of anaerobic respiration that occur in the rhizosphere. We also discuss the flexibility of anaerobic respiration as a fundamental trait for the microbial colonization of roots, environmental and ecological adaptation, persistence and bioremediation in the rhizosphere. Anaerobic respiration appears to be a key process for the functioning of an ecosystem and interactions between plants and microbes.

Keywords: rhizosphere competence, anaerobic respiration, terminal electron acceptors, respiratory pathways, rhizobacteria, root colonization, adaptation

INTRODUCTION

Bacteria are ubiquitous and occupy a very wide variety of ecological niches in particular the plant rhizosphere, the volume of soil influenced by root exudates (Kai et al., 2016). The rhizosphere is a densely populated soil fraction rich in carbon, insofar as 10–40% of photosynthetic fixed carbon is released through the root into the surrounding soil named “root-adhering soil” (Marschner, 1995; Haichar et al., 2014). As a result of this carbon flux, bacteria are attracted to the surface of the roots that they successfully colonize if they possess specific genetic and physiologic traits named “rhizosphere competence.” These traits shape bacterial growth and activity and are influenced by environmental factors, such as the pH, soil structure, water content, oxygen flux and nutrient availability (Philippot et al., 2013). Oxygen flux is one of the most changing factors in the rhizosphere, as plants can be subjected to aerobic or microaerophilic conditions followed by alternating exclusively anoxic conditions or anaerobic conditions, as in soil aggregates (Philippot et al., 1996). This variation in oxygen concentration constrains bacteria to adapt their physiology and to develop flexible metabolic pathways, such as the respiration of compounds other than oxygen when it is lacking. Indeed, bacterial anaerobic respiration is one of the most flexible and diverse metabolic processes (Hong and Gu, 2009). Among microbes, a wide range of compounds can serve as terminal electron acceptors (TEAs), including oxygen (the most energetic one); nitrogen oxyanions and nitrogen oxides (Zumft, 1997; Kuypers et al., 2018); carbon compounds, such as fumarate (Kröger, 1978; Alves et al., 2015); transition metals, such as Fe(III) and Mn(IV) (Lovley and Phillips, 1987; Kasai et al., 2015); metalloid oxyanions, such as selenate and arsenate (Macy et al., 1996; Switzer Blum et al., 1998; Glasser et al., 2018); radionuclides, such as U(VI) (Lovley et al., 1991; Marshall et al., 2006); and elemental sulfur and sulfur oxyanions (Klimmek et al., 1991; Eller et al., 2018). These TEAs are more or less all available in the rhizosphere and can serve for microbial anaerobic respiration. The appearance, development and evolution of such respiration have been the subjects of a numerous studies (Lovley and Coates, 2000; Richardson, 2000; Philippot, 2002; Kuypers et al., 2018). Respiratory chains have already been deeply studied in several bacteria (Richardson, 2000). Some bacteria present high anaerobic respiration flexibility through the use of different TEAs for growth. For example, *Deltaproteobacteria* exhibit considerable flexibility in respiratory processes, including sulfate reduction, iron reduction, fermentation, and dehalogenation (Sanford et al., 2002). Indeed, strains of *Geobacter* can at minimum use Fe(III), Mn(IV), nitrate, fumarate, arsenic oxyanions, and U(VI) as TEAs (Lovley et al., 1991). *Shewanella oneidensis* is even more versatile than *Geobacter* and can use additional TEAs, such as sulfur oxyanions and selenium oxyanions (Beliaev et al., 2005; Klonowska et al., 2005). Due to the high flexibility of anaerobic respiration, microorganisms affect a number of biogeochemical cycles, such as those for N, C, Fe and Se (Richardson, 2000). Those processes may result to greenhouse gases release (CO₂ and N₂O) and nutrient loading and consumption, which coupled with changes of interacting

spheres of the earth (Zheng et al., 2018). Globally, bacterial anaerobic respiration is well-known but remains an incomplete story in the rhizosphere. Indeed, aside from denitrification and Fe(III) respiration, little is known about other respiration processes, and only few model strains have been deeply studied, even if certain rhizospheric models possess genes involved in anaerobic respiratory pathways. Furthermore, the reasons for microbial respiratory diversity and flexibility in the rhizosphere has yet remain incompletely known, but this diversity appears key to rhizosphere competence, adaptation, and persistence belowground. For example, microbial respiratory flexibility is suggested to provide competitive advantage to Fe(III)-reducing bacteria to colonize iron oxide plaque on root surfaces (Somenahally et al., 2011) and confers rhizosphere competence to denitrifiers in colonizing different plant rhizospheres, such as those of maize and tomato plants (Philippot et al., 1995; Mirleau et al., 2001; Chèneby et al., 2004).

In this review, we detail for the first time, anaerobic respirations that occur in the rhizosphere. First, attention is paid to the environmental conditions that affect anaerobic respirations and the different respirations encountered in the rhizosphere. Second, research on the genes involved in anaerobic respiration from metatranscriptomic data on the rhizosphere are conducted to evidence how this trait is largely distributed among rhizobacteria. Finally, the ecological and environmental advantages of anaerobic respiration in the rhizosphere are discussed.

ANAEROBIC RESPIRATIONS IN THE RHIZOSPHERE

Plant roots modulate anaerobic respiration both indirectly and directly. Indeed, it is well-known that roots increase the anaerobic volume of the soil by consuming oxygen, exude different carbon sources that may act as TEAs, such as fumarate, and compete with the microbial community for certain elements, such as nitrate, thereby diminishing its availability to microbes. Therefore, elucidation of how the rhizospheric microbiota switches from aerobic to anaerobic respiration in the absence of oxygen and which alternative electron acceptors it uses holds great potential to understand population dynamics and evolutionary strategies for coping with oxygen depletion.

Environmental Conditions Affecting Anaerobic Respirations

Oxygen Flux

One of the most significant factors influencing anaerobic respiration is the lack of oxygen. Its flux has been extensively studied at the root level of submerged plants (Christensen et al., 1994). However, only a few studies are available regarding the rhizosphere. The spatial distribution of oxygen depends strongly on the investigated scale. At the pedon scale, a higher O₂ partial pressure (pO₂) is found in the topsoil and gradually decreases with depth (Stepniewski and Stepniewska, 2009) due to the greater diffusion distance from the free atmosphere. At the aggregate scale, pO₂ decreases from the outside perimeter

to the aggregate heart, which can reach anaerobic conditions (Sexstone et al., 1985; Zausig et al., 1993; Philippot et al., 1996). At the rhizosphere scale, O_2 distribution from the root surface into the bulk soil is driven by its consumption due to root and microbiotic respiratory processes and diffusive O_2 supply (Glinski and Stepniowski, 1985; Uteau et al., 2015). Oxygen flux in soil is very difficult to predict, as it depends on soil properties such as porosity, texture, organic matter content, moisture content, temperature and pH (Runkles, 1956). The soil structure is the most important soil property related to oxygen diffusion in the soil. Indeed, the modification of the soil structure due to compaction limits gas transport in the soil, since it decreases the pore spaces filled with air where gas diffusion occurs (Neira et al., 2015). In addition, soil organic matter impacts oxygen level by stabilizing soil aggregates (Uteau et al., 2013).

Terminal Electron Acceptors and Their Distribution in the Rhizosphere

The basic anaerobic respiratory process involves the oxidation of a low-redox-potential electrons donor, such as carbon substrates, the transfer of electrons through a wide range of cytochromes and the reduction of a high-redox-potential electrons acceptor. Bacteria synthesize ATP through a membrane-bound ATP synthase complex driven by proton-motive force with the intermediary action of cytochromes (Richardson, 2000; Kartal et al., 2011). A wide range of metallic and non-metallic terminal electron acceptors (TEAs) is encountered in soils and can serve for bacterial anaerobic respiration (Figure 1, Table 1). Their availability is spatio-temporally variable (Jackson and Caldwell, 1993; Farley and Fitter, 1999; Lark et al., 2004) and depends on the constant interaction of processes such as weathering, atmospheric deposition, nutrient leaching, biological cycling, and plants nutrition, resulting in the formation of vertical and horizontal TEA gradients within the soil (Figure 1) (Giehl and Wirén, 2014). Additional processes, such as rainfall, the inflow of dust in agricultural soils, and the supply of manure and chemical fertilizers, introduce additional nutrients to the soil pool (Giehl and Wirén, 2014), which can act as TEAs.

Non-metallic electron acceptors

Nitrate, fumarate, and humic substances represent the most studied non-metallic TEAs. Their availability in the rhizosphere is variable and depends on different biotic and abiotic factors. The bioavailability of N forms in soils, such as nitrate (NO_3^-) and nitrite (NO_2^-) is a dynamic balance influenced by microbial nitrification and denitrification (Jones and Hallin, 2018). NO_2^- formation occurs near the surface and NO_2^- concentration decrease rapidly with depth (Arth and Frenzel, 2000). The spatial and temporal availability of soil NO_3^- is highly heterogeneous. At the centimeter scale or over the course of a day, NO_3^- may vary by an order of magnitude (Beliaev et al., 2005; Klonowska et al., 2005). This heterogeneity is derived from several factors that release or remove nitrates from the soil (Bloom et al., 2002). For example, a high rate of nitrification activity or fertilizer use in agricultural soils leads to an increase in nitrate concentration (Burger and Jackson, 2003; Galloway et al., 2008), whereas plant root uptake, which competes with soil microbiota, reduces nitrate

in soils (Kuzakov and Xu, 2013). Notably, some bacteria impact positively N availability for plants, such as Rhizobia that are able to provide N_2 to plants by nitrogen fixation in nodules (Haichar et al., 2014). In the rhizosphere, nitrate move freely within the soil and reach the root surface through mass flow, which is driven by the nutrient concentration in the soil solution and the rate of plant transpiration (Giehl and Wirén, 2014). Their concentration varies from 0.04 to 5 g.kg⁻¹ for soils deficient in nitrate to 80–100 g.kg⁻¹ for soils rich in nitrate (unpublished data).

Fumaric acid or fumarate is an organic acid exuded by plants in the rhizosphere of various plant species such as maize, banana and *Dactylis glomerata* (Zhang et al., 2014; Guyonnet et al., 2017). Its concentration in the rhizosphere varies according to plant developmental stage (Petersen and Böttger, 1991). Recently, Guyonnet et al. (2017) evidenced that the relative abundance of fumaric acid was approximately 0.04% in the root-adhering soil of *D. glomerata*. Fumaric acid exudation was also observed with barley (*Hordeum vulgare* cv. Barke) and was likely involved in the tripartite interaction of beneficial microorganisms, plant and pathogens in the rhizosphere (Jousset et al., 2014). In addition, Liu et al. (2014) and Yuan et al. (2015) demonstrated that fumaric acid exudation is involved in improving plant growth-promoting rhizobacterial colonization by stimulating biofilm formation. Hence, fumaric acid has a role in plant-microbe interactions and in microbial anaerobic respiration in the rhizosphere.

In addition to fumaric acid, humic substances (HSs) have also been investigated as TEAs for microbial anaerobic respiration (Lovley et al., 1996; Hong and Gu, 2009). HS are highly polymerized natural polymers of high molecular weight and poor biodegradability that are formed during the decomposition of plant materials by microorganisms in soils and sediments (Hong and Gu, 2009). The remarkable recalcitrance of such materials is reflected by their long residence times in the environment, exceeding 500 years. Lovley et al. (1996) were the first to put forward the concept of humic respiration, which was supported by experiments in which both *Geobacter metallireducens* and *Shewanella alga* can grow with humus and/or anthraquinone-2,6-disulfonate (AQDS) as TEAs when a wide variety of organic substrates or H_2 are oxidized.

Metallic electron acceptors

The most-studied metallic TEA are iron [Fe (III)], manganese [Mn (IV)], arsenic [As (V)], selenium [Se (VI)], sulfur (S^0), and uranium [U(VI)]. Their availability in the rhizosphere also depends on biotic and abiotic factors. Among those TEAs, Fe, Mn, and S also serve as plant nutrients (Giehl and Wirén, 2014); hence, mutualistic/competitive interactions may occur with rhizosphere microbiota for their uptake (Somenahally et al., 2011; Moreau et al., 2015).

Iron (Fe) is a relatively abundant element in many cultivated soils, with an average total concentration of approximately 20–40 g.kg⁻¹ (Cornell and Schwertmann, 2003); however, the amount of its available fraction is generally very small (Colombo et al., 2014). Iron is considered an essential micronutrient for plants and microbes, and its availability is crucial for their growth. Thus, bacteria and plants both adopt strategies to acquire Fe

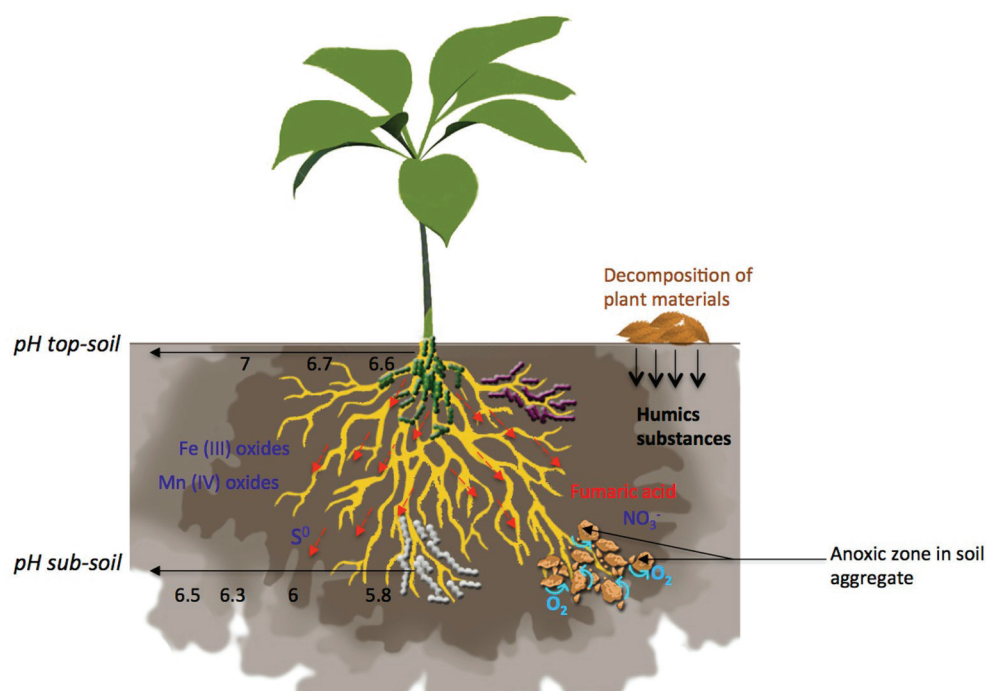


FIGURE 1 | Terminal Electron Acceptors (TEAs) distribution, O_2 flux and pH gradient in the rhizosphere. Many factors are involved in the formation of zones favorable for anaerobic respiration establishment such as TEAs availability, pH, water movement and soil aggregate formation. NO_3^- moves freely within the soil, with a vertical gradient of availability from sub-soil to top-soil. Decomposition of plant materials leads to the release of humic substances. Fumaric acid is exuded by plant roots. Availability of Fe(III), Mn(IV) and S^0 is mostly driven by pH. Roots influence pH leading to a gradient from an acidic pH close to the roots to a neutral pH in the rhizosphere. Anoxic zones occur in soil aggregates and in soils submitted to water flow.

for their metabolisms, such as the secretion of microbe- and phyto-siderophores (Colombo et al., 2014). Fe availability in the rhizosphere is mediated by two major factors: the redox potential (*i.e.*, oxidizing or reducing conditions) and pH (Lemanceau et al., 2009; Colombo et al., 2014). In fact, soil pH can increase or decrease by up to two units in the rhizosphere owing to the release and uptake of ions by roots (Hinsinger et al., 2009). Among Fe species present in the soil environment, a wide variety of Fe(III) oxides and clay minerals can be used as TEAs during microbial anaerobic respiration (Lovley et al., 2004; Weber et al., 2006; Colombo et al., 2014).

Manganese is, an essential trace element for life, usually present in relatively large amount in the plant rhizosphere (Uren, 2013), and its availability is governed by oxidation and reduction processes (Marschner, 1988; Posta et al., 1994). The balance between these two processes is a function of soil chemical, microbial and plant factors (Marschner, 1988). Theoretically, Mn may exist in any of the redox states ranging from 0 to +7, but it is primarily present under the three oxidation states +II, +III, and +IV in natural environments (Davison, 1993). In addition, various oxides and oxyhydroxides of Mn(IV) are present in soils and serve as sinks for bioavailable Mn (Post, 1999). These Mn oxides are typically generated during diagenetic processes or the oxidation of Mn(II) (Mouret et al., 2009) and serve as TEAs for microbial anaerobic respiration (Lovley et al., 2004).

In soils, sulfur occurs in inorganic and organic forms and is cycled between these forms *via* mobilization, mineralization, immobilization, oxidation, and reduction processes (Scherer, 2009). Whereas, organic sulfur compounds are largely immobile, inorganic sulfur is more mobile, with sulfate being the most mobile (Scherer, 2001). Sulfate is also the most important S source for plants, generally accounting for <5% of the total S in the soil (Scherer, 2009). In grassland soils, the total S content in the upper soil layer (0 to 20 cm) ranges from 0.21 to 1.7 g.kg⁻¹ (Wang et al., 2006). Among sulfur compounds encountered in soils, sulfite, organic sulfoxides, elemental sulfur, polysulfide, tetrathionate, and organic disulfides are important TEAs in the biogeochemical cycling of sulfur anaerobically (Schauder and Kröger, 1993; Kertesz, 2000; Burns and DiChristina, 2009). Their availability, as for Fe(III) oxides, is also dependent on pH (Hedderich et al., 1998).

Selenium is an essential trace element available in different chemical and physical forms and is found in all natural environments, such as soils (Nanchaiah and Lens, 2015). In most soils, selenium concentrations are very low, in the range of 0.01–2 mg.kg⁻¹. However, in seleniferous soils, selenium concentrations of up 1,200 mg.kg⁻¹ have been reported (Fordyce, 2013). The selenium cycle has a wide range of oxidation states, from –II to +VI, and their distribution may vary in the environment depending on the prevailing redox conditions

TABLE 1 | Characteristics of all anaerobic respiration reviewed in this study.

| | Nitrate | Fumarate | Humic acids | Fe(III) | Mn(IV) | U(VI) | SeO ₄ ²⁻ | SeO ₃ ²⁻ | As(V) | S ⁰ |
|-------------------------------------|--|--|---|--|--------------------------------|---|---|---|---------------------------------|--|
| Availability in soil / rhizosphere | 0.04 to 100 g.kg ⁻¹ soil | ND | ND | 20 to 40 g.kg ⁻¹ soil | 1 to 3 g.kg ⁻¹ soil | 8.1 × 10 ⁻⁴ to 3.2 μg.g ⁻¹ soil | 0.01 to 2 mgSeO ₄ .kg ⁻¹ soil | 0.01 to 2 mgSeO ₃ .kg ⁻¹ soil | 2 to 3 mg.kg ⁻¹ soil | 0.04 to 2.7 g.kg ⁻¹ soil |
| ΔG°' (kJ.mol ⁻¹ acetate) | -802 | -249.3 | ND | -717 | -727 | ND | -575 | -529.5 | -252.6 | -39 |
| Genes involved | <i>narG</i> , <i>napA</i> , <i>nirK</i> , <i>nirS</i> , <i>norCB</i> , <i>nosZ</i> (1) | <i>frdABCD</i> (<i>E. coli</i>) (2) <i>cymA</i> (<i>S. oneldensis</i> MR-1) (3) | <i>omcB</i> STEZ (<i>G. sulfurreducens</i>) (4) | <i>mirCAB</i> , <i>omcA</i> , <i>cymA</i> (<i>S. oneldensis</i> MR-1) (5) | | | <i>serABC</i> (7) | <i>fcaA</i> ? (<i>S. oneldensis</i> MR-1) (7) | <i>arrAB</i> (7) | <i>psrABC</i> (<i>W. succinogenes</i>) (8) |

The availability of TEAs found in the soil or the rhizosphere, the free energy generated through reduction of these TEA and the genes involved in these anaerobic respirations are summarized here. No data (ND) on free energy release during humic substances and U(VI) respirations is available. (1) Zumft, 1997; (2) Jones and Gunsalus, 1987; (3) Myers and Myers, 1997; (4) Voordeckers et al., 2010; (5) Breischger et al., 2007; Marshall et al., 2006; (6) Akujkar et al., 2013; (7) Stolz et al., 2006; (8) Hedderich et al., 1998.

and pH (González et al., 2006). Generally, selenium oxyanions selenate [SeO₄²⁻ or Se (VI)] and selenite [SeO₃²⁻ or Se (Se IV)] are soluble and stable and exhibit high bioavailability and toxicity in oxic environments. In anoxic conditions, selenium oxyanions serve as TEAs for anaerobic microbial respiration and are replaced by elemental selenium (Se⁰).

Arsenic, despite its toxicity, is readily used by a great diversity of prokaryotes for cell growth and metabolism (Stolz et al., 2006). Arsenic, as is the case with selenium, is widely distributed in the environment, with an average As content in Earth's crust varying between 2 and 3 mg.kg⁻¹ (Tanaka, 1988). Its concentration in soils depends on rock type, human activities, climate, As forms and soil redox conditions (Yan-Chu, 1994). Arsenic is found in four oxidation states, arsenate As(V), arsenite As(III), elemental As⁰ and arsenide As(-III) distributed according to oxygen availability (Stolz et al., 2006; Mohan et al., 2007). Among these species, As(V) is used as a TEA by microbes for anaerobic respiration called “arsenate respiration,” which is the dominant process in anaerobic conditions (Oremland et al., 2005).

Uranium is an important component of Earth's crust and is found in all environments from water to soils (Gavrilescu et al., 2009). Uranium is a radioactive element whose 3 dominant isotopes are ²³⁸U, ²³⁵U, and ²³⁴U. Thus, uranium is a toxic metal, and multiple studies are interested in the remediation of this element (Akob et al., 2007; Lee and Yang, 2010; Choudhary and Sar, 2011; Yan et al., 2016). Uranium is found in soil in concentrations ranging from 8.1 × 10⁻⁴ to 3.2 μg.g⁻¹ soil (Tzortzis and Tsertos, 2004). It is available in the environment in the form of oxides such as uranyl ion (UO₂²⁺) and UO₂OH⁺, which can be complexed with carbonate, nitrate or humic substances, for example, (Markich, 2002; Gavrilescu et al., 2009) and which can be used as TEAs for microbial anaerobic respiration.

As described above, the rhizosphere contains a large variety of compounds that can serve as terminal electron acceptors for microbial respiration in the anoxic zone of the rhizosphere. However, only a few studies have been interested in understanding anaerobic respiration in the rhizosphere (Philippot et al., 1995; Ghiglione et al., 2000; Mirleau et al., 2001). Denitrification represents the most studied one area, likely due to its large contribution to the increase of N₂O emissions, a gas that has an approximately 300-fold greater impact on global warming than CO₂ (Bakken et al., 2012; Daigneault-Horta et al., 2015; Gaimster et al., 2018). In the next paragraph, we review the different mechanisms of anaerobic respiration encountered in the environment and we attempt to determine whether certain respiration processes studied in a given environment can take place in the rhizosphere by the microbiota associated with plants.

The Different Types of Anaerobic Respiration in the Rhizosphere

In anoxic conditions, bacteria have developed specific processes that allow them to use sufficiently abundant alternative TEAs to survive, proliferate and acclimate quickly in a rapidly changing environment such as the rhizosphere. Indeed, the rhizosphere can lead to prolonged anoxic conditions such as in rice field

or aerobic conditions followed by alternating anoxic conditions such as soils aggregates (**Figure 1**) (Philippot et al., 1996; Kostka et al., 2002). In these conditions, the TEAs described above can be respired by rhizospheric microbiota. According to the acceptor, bacteria can grow more quickly, such as with nitrate, or less quickly, such as with sulfur (Nealson et al., 1994), due to the difference in free energy released during respiratory metabolism. Here, we summarize the different respiration processes, which potentially occur in the rhizosphere, and the energy they generated (**Figure 1**).

Denitrification

Denitrification is a part of nitrogen cycle and occurs in anaerobic conditions mainly by bacteria. Denitrification uses nitrate as a TEA and yields the highest energy to sustain growth among anaerobic respirations; it includes nitrate, nitrite, nitric oxide and nitrous oxide reductions ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$) (Zumft, 1997). Each reaction is catalyzed by a reductase that is coupled to energy-conserving electron-transport pathways and regulated by the transcriptional regulator “Fnr” (fumarate and nitrate reductase). Fnr is part of a superfamily of Crp-Fnr transcriptional regulators that includes Crp (catabolite repression), FnrN (nitrogen fixation/photosynthesis), FixK (nitrogen fixation), NnrR (response to NO in denitrification) and Dnr (dissimilatory nitrate respiration regulator) (Körner et al., 2003). Notably, in *Bradyrhizobium japonicum*, the global response regulator that controls expression of denitrification gene is called RegR (Torres et al., 2014).

Unlike other anaerobic respirations, genes encoding the denitrifying reductases have been characterized in bacteria inhabiting the rhizosphere, such as *Pseudomonas stutzeri*, *Pseudomonas fluorescens* and *Paracoccus denitrificans* (Zumft, 1997; Kuypers et al., 2018). The reductases involved in this respiratory process are described as dissimilatory nitrate reductase (membrane-bound “NAR, *narGH*” and periplasmic “NAP, *napA*”), nitrite reductase (Nir), nitric oxide reductase (cytochrome c-dependent “cNOR, *cnorB*”, quinol-dependent “qNOR, *norZ*” and copper-containing quinol-dependent nitric oxide reductases “Cu_ANOR”) and nitrous oxide reductase (Nos) (Philippot, 2002; Kuypers et al., 2018). Two types of dissimilatory nitrate reductases are found in bacteria and even in the same bacteria as in *Escherichia coli*: a membrane-bound (Nar) and a periplasmic-bound (Nap) nitrate reductase (Moreno-Vivián et al., 1999; González et al., 2006; Kraft et al., 2011). Nar is composed of 3 subunits encoded by *narGHI*, as found in *E. coli*, *P. fluorescens*, *P. stutzeri* and *B. subtilis* (Philippot and Højberg, 1999; Lalucat et al., 2006), whereas Nap is composed of 2 subunits encoded by *napAB*, as found in *Rhodobacter sphaeroides* and *B. japonicum* (Reyes et al., 1998; Bedmar et al., 2005). The *nar* operon is induced under low oxygen partial pressure and in the presence of nitrogen oxides (Philippot et al., 2001; Giannopoulos et al., 2017), whereas the expression of the *nap* operon can be affected or not by anaerobiosis depending on the organism (Philippot, 2002; Bueno et al., 2017).

A recent study demonstrated that the two types of nitrite reductase can also be found in the same bacteria, contrary to what has been previously accepted (Sánchez and Minamisawa,

2018). The two nitrite reductases encoded by *nirK* and *nirS* are evolutionarily unrelated enzymes (Philippot, 2002). Both nitrite reductases encoded by *nirS* in *P. fluorescens* and *P. stutzeri* and *nirK* in *R. sphaeroides* are expressed under low O₂ levels and in the presence of nitrate or nitrite (Tosques et al., 1997; Härtig and Zumft, 1999; Philippot et al., 2001). Moreover, it has been demonstrated that NO induces *nirS* expression in *P. stutzeri* (Vollack and Zumft, 2001). Nitric oxide reductase is composed of 2 subunits encoded by *norCB* and is expressed in the absence of O₂ and in the presence of NO (Zumft, 1997; Vollack and Zumft, 2001; Hino et al., 2010). The enzyme involved in nitrous oxide respiration is encoded by *nosZ* (Zumft and Kroneck, 2007; Hartsock and Shapleigh, 2010; Thomson et al., 2012). This gene is lacking in many denitrifying bacteria and archaea, leading to greenhouse gas emissions (Hu et al., 2015). Recently, it has been demonstrated that non-denitrifying bacteria, such as *Wolinella succinogenes*, can grow with N₂O as the sole electron acceptor, thereby allowing to the reduction of the N₂O greenhouse gas (Simon et al., 2004). These N₂O-reducing microorganisms are the subject of a recent review by Hallin et al. (2017) in which the two distinct groups of N₂O reducers and their ecology are described. They underscore the fact that NirK denitrifying microorganisms are more lacking the *nos* cluster than are NirS denitrifying microorganisms. So far, no study has been conducted on the co-occurrence of the *nar/nap* operons and *nirK* and/or *nirS* denitrifying microorganisms.

Anammox

Microorganisms contribute to N cycle not only through denitrification process also through anaerobic ammonium oxidation (anammox) process (van de Graaf et al., 1995). Anammox is responsible for a loss of total N from fertilized soils, such as paddy soils (Nie et al., 2015) but less significant than loss caused by denitrification. Anammox is the oxidation of ammonium to dinitrogen gas under anaerobic conditions with nitrite as TEA (Gori et al., 2011). Hydrazine (N₂H₄) and NO are intermediates of anammox process (Strous et al., 2006). Anammox activity is linked to the ammonium, nitrate and nitrite concentration in soils (Hu et al., 2011). So, this process depends on denitrification and ammonia oxidation rates to form the substrates of the reaction (Naeher et al., 2015). Anammox is also influenced by oxygen flux, soil pH, salinity and rhizosphere effect (Nie et al., 2018). Indeed, soil pH affects ammonium availability and hence plays an important role in shaping anammox bacterial community. Like denitrification, anammox respiration occurs in a larger variety of niches in rhizospheres and soils (Humbert et al., 2010; Li et al., 2016). The five predominant anammox bacterial genera are *Kuenenia*, *Brocadia*, *Jettenia*, *Scalindua*, and *Anammoxoglobus* and detected in many habitats, including soils and sediments (Humbert et al., 2010; Li et al., 2016). Genes encoding specific proteins involved in anammox were deciphered in *Kuenenia stuttgartiensis* (Strous et al., 2006; Kraft et al., 2011). The first step of anammox corresponding to the reduction of NO₂⁻ in NO is performed by the denitrification protein NirS. The second step of the predicted anammox pathway would be the reduction of NO and its simultaneous condensation with ammonium to produce N₂H₄. In *K. stuttgartiensis*, two

gene clusters potentially involved in this step may formed an enzymatic complex called hydrazine synthase, HZS (Kraft et al., 2011). The final step of annamox is the oxidation of N_2H_4 to N_2 . In *K. stuttgartiensis*, the dedicated enzyme involved in this step is the hydrazine dehydrogenase (HDH) encoded by *kuste0694* (Kraft et al., 2011).

Anammox respiration is a sufficient energetic strategy to sustain growth during anaerobic conditions (Kraft et al., 2011). The overall reaction for anammox is $\text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2 \text{H}_2\text{O}$ and the associated free energy is -358 kJ.mol^{-1} of NH_4^+ (van de Graaf et al., 1995, 1996).

Fe(III) and Mn(IV) Respiration

Electron acceptors such as Fe(III) and Mn(IV) are highly insoluble in most environments at pH 7. Thus, there is an important challenge for Fe(III) and Mn(IV) reducers to develop strategies for electron transfer to an insoluble and extracellular TEA (Lovley et al., 2004). Dissimilatory Fe(III)-reducing bacteria need (i) to transfer electrons from the central metabolism to a site of reduction somewhere outside the inner membrane and (ii) to express a reductase located on the outer membrane to reduce Fe(III) oxides to Fe (II). The same observations can be made for Mn (IV)-respiring bacteria (Lovley et al., 2004).

Fe (III) and Mn (IV) anaerobic respirations have been extensively studied in *S. oneidensis* MR-1 and *Geobacter sulfurreducens* models (Lovley et al., 2004). However, until recently, no study has been made available using rhizobacteria as model. In addition, the mechanisms responsible for these respirations are not fully understood, but a number of genes seem to be involved (Bretschger et al., 2007). In *S. oneidensis* MR-1, these include the *mtrA*, *mtrB*, *mtrC*, *omcA* and *cymA* genes. MtrABC serve as an electron conduit between the periplasm and the extracellular environments (Hartshorne et al., 2009). MtrC and OmcA have been hypothesized to serve as terminal Mn(IV) and Fe(III) reductases (Bretschger et al., 2007). CymA, cytoplasmic membrane-bound, tetraheme c-type cytochrome, is involved in electron flow during Fe(III) and Mn (IV) respiration (Myers and Myers, 1997). In *G. sulfurreducens*, different genes are

also involved in each respiratory pathway (Aklujkar et al., 2013). Interestingly, OmcS, an outer-membrane protein, is required for both types of respiration (Mehta et al., 2005). Respiration of Fe(III) or Mn(IV) has the potential to yield sufficient energy to support growth, as does nitrate (Tables 1, 2). Interestingly, some anammox bacteria, such as *K. stuttgartiensis* are known to use Fe(III) and Mn(IV) oxides as TEA during anaerobic conditions (Strous et al., 2006; Zhao et al., 2014).

Fumarate Respiration

Fumarate respiration has been well-known since the 1970s. However, it has been described in only a few bacteria, such as *E. coli*, *W. succinogenes* and *Shewanella* (Ackrell et al., 1992; Kröger et al., 1992; Arkhipova and Akimenko, 2005). Fumarate is reduced to succinate, the reverse reaction of succinate to fumarate that occurs aerobically in the tricarboxylic acid cycle

(TCA cycle). Under anaerobic conditions in the presence of fumarate in *E. coli*, a specific membrane quinol-bound fumarate reductase complex encoded by four genes *frdA-D* is induced (Jones and Gunsalus, 1987). Fumarate reductase is a distinct complex from the succinate dehydrogenase complex, encoded by *sdhA-D* genes. The regulation of these two enzymes is quite distinct since *frd* genes are expressed under anaerobic conditions in the presence of fumarate, whereas *sdh* genes are expressed under aerobic conditions and depend on glucose (Maklashina et al., 2013). In addition to the fumarate reductase complex, different global regulators are also involved in fumarate respiration, such as FNR and CymA. Indeed, in *S. oneidensis* MR-1, as in Fe(III) oxides and Mn(IV) respirations, CymA is also involved in anaerobic fumarate respiration (Myers and Myers, 1997).

Unlike the aforementioned respiration processes, fumarate respiration is less energetically favorable (see Tables 1, 2).

Sulfur Respiration

Anaerobic respiration with elemental sulfur/polysulfide or organic disulfides is performed by several bacteria and archaea but has only been investigated in detail in a few organisms, such as *W. succinogenes* and *S. oneidensis* MR-1. The electron transport

TABLE 2 | Comparison of free energy $\Delta G^{\circ'}$ for various TEAs coupled to acetate oxidation and associated reactions.

| Reaction | Free energy $\Delta G^{\circ'}$ (kJ.mol ⁻¹ acetate) | References |
|---|--|---------------------------|
| $5 \text{CH}_3\text{COO}^- + 8 \text{NO}_3^- + 13 \text{H}^+ \rightarrow \text{N}_2 + 10 \text{CO}_2 + 4 \text{N}_2 + 14 \text{H}_2\text{O}$ | | Thauer et al., 1989 |
| $\text{CH}_3\text{COO}^- + 4 \text{MnO}_2 + 2 \text{CO}_2 + \text{H}^+ \rightarrow 4 \text{MnCO}_3 + 2 \text{H}_2\text{O}$ | -802 | Thauer et al., 1989 |
| $\text{CH}_3\text{COO}^- + 24 \text{Fe(OH)}_3 + \text{H}^+ \rightarrow 8 \text{Fe}_3\text{O}_4 + 2 \text{CO}_2 + 38 \text{H}_2\text{O}$ | -727 | Thauer et al., 1989 |
| $\text{CH}_3\text{COO}^- + 4 \text{SeO}_4^{2-} + \text{H}^+ \rightarrow 4 \text{SeO}_3^{2-} + 2 \text{CO}_2 + 2 \text{H}_2\text{O}$ | -717 | Nanchaiah and Lens, 2015 |
| $\text{C}_2\text{H}_4\text{COO}^- + \text{SeO}_3^{2-} + \text{H}^+ \rightarrow \text{CH}_3\text{COO}^- + \text{Se}^0 + \text{HCO}_3^- + \text{H}_2\text{O}$ | -575 | Nanchaiah and Lens, 2015 |
| $\text{CH}_3\text{COO}^- + 2 \text{HAsO}_4^- + 2 \text{H}_2\text{AsO}_4^- + 5 \text{H}^+ \rightarrow 4 \text{H}_3\text{AsO}_3 + 2 \text{HCO}_3^-$ | -529.5 | Macy et al., 1996 |
| $\text{CH}_3\text{COO}^- + 4 \text{fumarate}^{2-} + \text{H}^+ \rightarrow 4 \text{succinate}^{2-} + 2 \text{CO}_2 + 2 \text{H}_2\text{O}$ | -252.6 | Yoon et al., 2013 |
| $\text{CH}_3\text{COO}^- + 4 \text{S}^0 + \text{H}^+ + 2 \text{H}_2\text{O} \rightarrow 4 \text{H}_2\text{S} + 2 \text{CO}_2$ | -249.3 | Thauer et al., 1989 |
| $\text{CH}_3\text{COO}^- + 4 \text{U(VI)} + 4 \text{H}_2\text{O} \rightarrow 4 \text{U(IV)} + 2 \text{HCO}_3^- + 9 \text{H}^+$ | -39 | Lovley et al., 1991 |
| | ND | |
| | Free energy $\Delta G^{\circ'}$ (kJ.mol ⁻¹ NH_4^+) | |
| $\text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2 \text{H}_2\text{O}$ | -358 | van de Graaf et al., 1995 |

The reactions are arranged in energetic order. ND for no data available.

chain that catalyzes the polysulfide reduction in *W. succinogenes* consists of polysulfide reductase (Psr) which is composed of 3 subunits (PsrA, B, C) encoded by *psrABC* genes, where PsrA seems to be the catalytic subunit (Hedderich et al., 1998), PsrB serves for electron transfer, and PsrC anchors the polysulfide reductase (PsrA) (Krafft et al., 1995).

The energy gain from sulfur respiration is the smallest compared to the previous respiration processes (Table 2; Thauer et al., 1989). The data suggest that this respiration process does not support bacterial growth and probably allows bacteria to persist until conditions are favorable.

Selenium Oxyanion Respiration

Dissimilatory reduction of selenium oxyanions, viz., selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), is significantly important in the environment and involves the conservation of metabolic energy for microorganisms (Knight et al., 2002). Microbes that can reduce selenium oxyanions are not restricted to any particular group or subgroup of prokaryotes, and examples are polyphyletically found throughout the bacterial and archaeal domain (Watts et al., 2003). Selenium oxyanion respiration consists of 2 distinct respirations: selenate and selenite. Two successive reductions occur during anaerobic respiration: selenate to selenite and selenite to selenium. Anaerobic respiration of selenate is induced by its presence under anaerobic conditions, leading to the formation of selenite and then selenium (Debieux et al., 2011). It has been suggested that selenate reduction may be catalyzed in many cases by bacterial nitrate reductases, and selenate reductase activity of both membrane-bound nitrate reductase (NAR) and periplasmic nitrate reductase (NAP) has been reported (Avazéri et al., 1997; Sabaty et al., 2001). However, it is evident that nitrate reductases are poor reducers of selenate and may not contribute significantly to global selenate reduction, particularly in areas enriched with both selenate and nitrate (Watts et al., 2003). Consequently, novel enzyme systems that catalyze the reduction of selenate selectively have been sought, and to date, detailed biochemical studies have been limited mainly to species isolated from aqueous or contaminated environments, such as *Thauera selenatis*, *Bacillus selenatarsenatis* SF-1, *Sulfurospirillum barnesii*, and *Enterobacter cloacae* SLD1a-1 (Rabenstein and Tan, 1988; Losi and Frankenberger, 1997; Yamamura and Amachi, 2014). For example, the selenate reductase of *T. selenatis* is part of the *SerABC* operon (Rabenstein and Tan, 1988), whereas the selenate reductase respiratory activity in *B. selenatarsenatis* is conferred by the *srdBCA* operon (Kuroda et al., 2011). In *T. selenatis*, *SerABC* has a high affinity to selenate and does not use selenite or other electron acceptors as substrates (Stolz et al., 2006). In addition, *SerABC* shares several similarities with the respiratory nitrate reductases. Furthermore, genetic studies have shown that selenate reductase activity requires the global anaerobic regulatory gene FNR (Yee et al., 2007).

At present, no gene has been identified for the second reduction of selenate respiration from selenite into elemental selenium in *T. selenatis*. Anaerobic respiration of selenite is induced by its presence under anaerobic conditions, leading to the formation of S^0 . It is well-established that selenite

reduction can be categorized into anaerobic respiration or a microbial detoxification strategy (Turner et al., 1998). The genetic mechanism of selenite respiration has been investigated only in *S. oneidensis* MR-1, and some mutagenesis analyses have been performed on respiratory or trans-membrane reductase (Taratus et al., 2000). It is still unclear whether *S. oneidensis* MR-1 is able to grow on selenite as the sole electron acceptor or if it is just a fortuitous detoxification process during anaerobic growth on fumarate, where *fccA* gene may be involved (Li et al., 2014).

Arsenic Oxyanion Respiration

Arsenic respiration has been well-studied in Mono Lake, resulting in the isolation of a number of arsenate-respiring bacteria (Oremland et al., 2000; Hollibaugh et al., 2005). The respiratory arsenate reductase (Arr) from *Chrysiogenes arsenatis*, *Bacillus selenitireducens*, *Shewanella*, and *Alkalilimnicola ehlichii* was identified and characterized (Krafft and Macy, 1998; Afkar et al., 2003; Richey et al., 2009). The arsenate reductase is a heterodimer, with a large catalytic subunit (ArrA) and a smaller electron transfer protein (ArrB). Stolz et al. (2006) performed a phylogenetic analysis of available sequences from arsenate reductases (Arr) and showed a high similarity between them. Remarkably, a complete genome analysis of *A. ehlichii* indicated that it lacks a conventional arsenite oxidase, but it instead possesses two operons that each encodes a putative respiratory arsenate reductase (Arr) expressed under different conditions acting as a bidirectional enzyme (Richey et al., 2009).

Uranium Respiration

Only few known strains are able to anaerobically reduce uranium to sustain their growth such as *Shewanella putrefaciens* strain 200, *S. oneidensis* MR-1, *G. metallireducens*, and *Desulfotomaculum reducens* (Lovley et al., 1991; Tebo and Obraztsova, 1998). The reduction of U(VI) anaerobically was particularly deeply investigated in both *Shewanella* strains (Lovley et al., 1991; Wade and DiChristina, 2000; Marshall et al., 2006). To date, no uranium-specific reductase has been reported, but certain *c*-type cytochromes implicated in Fe(III) and Mn(IV) anaerobic respirations have been found to be involved in uranium reduction, such as MtrA, MtrB, MtrC, and OmcA (Beliaev and Saffarini, 1998; Beliaev et al., 2001; Marshall et al., 2006). Notably, in *S. putrefaciens* strain 2000, anaerobic respiration of U(VI) seems to be related to denitrification, as the U(VI) reduction-deficient mutant (Urr) lacks the ability to growth using U(VI) and nitrite.

Humic-Substance Respiration

Humic substances (HSs) are the major component of soil's organic matter and are derived from the partial degradation of plant material (Trevisan et al., 2010). The biogeochemical cycle of HSs is driven through oxidation and reduction performed by various microorganisms that can use HSs as electron donors and/or acceptors. Even if HS respiration has been known since the 1990s (Lovley et al., 1996), only few models have been studied for their ability to reduce HSs, such as *Shewanella* and *G. sulfurreducens* (Lloyd et al., 2003; Hong et al., 2007). Certain genes involved in this respiration process were evidenced

in *G. sulfurreducens* (Voordeckers et al., 2010) through the mutagenesis of five *c*-type cytochromes, OmcB, OmcS, OmcT, OmcE, and OmcZ. The authors concluded that AQDS and HS reduction might occur at the outer surface through these five *c*-type cytochromes. It is well-accepted that priming the soil with HS causes an increase in microbial activity due increased availability of energy required by microbes (Shah et al., 2018). However, to our knowledge, no information is available on the energy yielded by the anaerobic reduction of HSs.

As seen above, some bacteria are able to respire several TEAs, such as *S. oneidensis* or *G. sulfurreducens*. Remarkably, electron flows are shared by several anaerobic respiration processes. Indeed, the Mtr pathway of *S. oneidensis* is involved at least in Fe(III), Mn(IV) and U(VI) respiration (Beliaev et al., 2005; Marshall et al., 2006). Thus, in the presence of multiple TEAs in a complex environment, such as the rhizosphere in absence of O₂, microorganisms will use TEAs in specific order (Achtnich et al., 1995). Even if this order is specific to each microorganism, they generally prefer to use the more energetic option first (Achtnich et al., 1995; Table 2).

Anaerobic Respiration: A Trait Largely Distributed Among Rhizospheric Bacteria

As described below, the majority of anaerobic respiration models leverage aqueous environments or paddy-rice fields (Somenahally et al., 2011), with only a few having been derived from the plant rhizosphere, except for denitrification respiration (Zumft, 1997).

Here, to evidence whether genes involved in iron, sulfur, selenate and arsenate respirations are expressed in the plant rhizosphere, we used available rhizosphere metatranscriptomic data for BLAST analysis. Ten protein sequences from 6 model strains were selected as templates: MtrC and OmcA from *S. oneidensis* MR-1 for Fe(III) respiration; PsrA from *W. succinogenes* and *S. oneidensis* MR-1 for sulfur respiration; ArrA from *Shewanella* ANA-3, *B. selenitireducens*, and *C. arsenatis* for arsenate respiration; and SerA, SerB, and SerC from *T. selenatis* for selenate respiration. Eleven rhizosphere metatranscriptomic bioprojects published on NCBI (<https://www.ncbi.nlm.nih.gov/sra/docs/>), retrieved from the rhizospheric soil and/or the roots of *Citrus* (Zhang et al., 2017), *Arabidopsis thaliana*, *Vellozia epidendroides*, *Populus*, *Miscanthus*, corn, switchgrass, maize, canola, and sorghum were analyzed. The raw reads from these data were filtered, trimmed, and quality-controlled to generate the unique and clean reads. Then, we compared the sequence similarity by BLAST analysis (at least 40% identity and 80% coverage) of reference protein sequences against these unique reads. Interestingly, we found that genes involved in the selected anaerobic respirations are expressed in the rhizospheres of *Citrus*, *Arabidopsis thaliana*, *Vellozia epidendroides*, *Populus*, *Miscanthus*, corn, switchgrass and sorghum, confirming that the anaerobic respiration of iron, sulfur, selenate and arsenate occur in the plant rhizosphere and that such an environment is favorable for the establishment of these respiration processes by the rhizospheric microbial community (Figure 2). Notably,

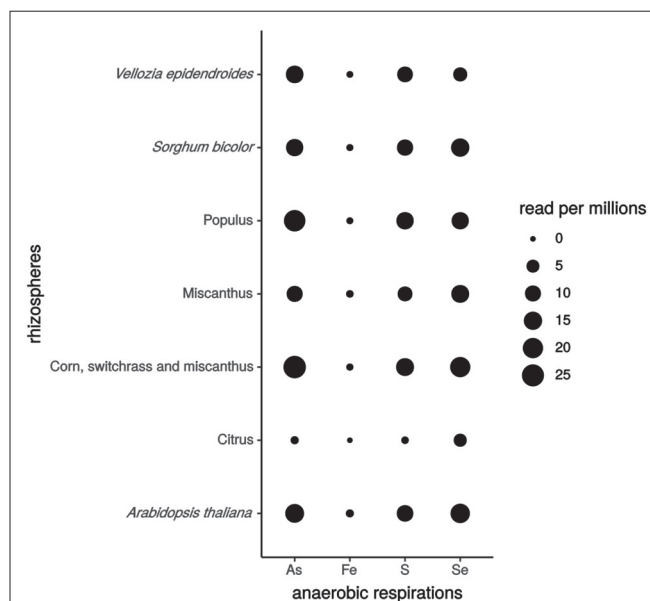


FIGURE 2 | Anaerobic respiration gene expression in different plant rhizospheres. The number of hits per 10⁶ reads expressed the level of genes expression. As for Arsenate, Fe for iron, S for sulfur and Se for selenate. Rhizospheric metatranscriptomics bioprojects accession numbers: *Vellozia epidendroides* (PRJNA441428), *Sorghum bicolor* (PRJNA406786), *Populus* (PRJNA375667), *Miscanthus* (PRJNA337035), [corn, switchgrass and *Miscanthus*] (PRJNA365487), *Citrus* (PRJNA324090), *Arabidopsis thaliana* (PRJNA366978, PRJNA366977, PRJNA336798).

genes encoding Fe(III) respiration enzymes were less expressed in the various plant rhizospheres tested, suggesting that this is due either to a lower conservation of these genes between *S. oneidensis* MR-1 and rhizobacteria, or to Fe oxides being less available for anaerobic respiration.

Further studies are needed to identify and isolate model strains of the rhizosphere expressing anaerobic respiration genes to better elucidate the importance and extent of anaerobic respiration processes in this environment. We suggest that microbial anaerobic respiration appears to be a key process for ecosystem functioning and plant-microbe interactions that need to be put in the spotlight.

ECOLOGICAL AND ENVIRONMENTAL SIGNIFICANCE OF ANAEROBIC RESPIRATION

Does the Anaerobic Respiration of Available TEAs Confer Competitive Advantage for Plant Colonization?

As reported above, rhizobacteria present a flexible respiratory process that may allow them to colonize and acclimate rapidly to a changing environment represented by the rhizosphere, where oxygen can be rapidly limited. In addition, certain respirations, such Fe(III) oxides by Fe(III)-reducing bacteria (FeRB), participate in Fe(II) availability for plants. Consequently,

under anaerobic conditions and in the presence of Fe(III) oxides, FeRB are more abundant on the root surface of plants, as shown by Somenahally et al. (2011), suggesting a specific selection of this functional group by plants on the root surface, the most selective and rich habitat (Haichar et al., 2008). Under anaerobic conditions, Fe(III) oxide respiration may be considered a trait that allows bacteria to colonize plant roots, and a mutualistic interaction may take place whereby the plant provides root exudates for bacterial growth while the bacteria in participating iron availability for the plant.

With regard to iron, performing a denitrification process also offers competitive advantage for bacteria to colonize plant roots (Chèneby et al., 2004). Indeed, several authors have reported on the selection of the denitrifying community by plant roots (Henry et al., 2008; Guyonnet et al., 2017). However, only a few studies have evaluated the role of denitrification's function in the selection of microorganisms in the rhizosphere (Philippot et al., 1995; Ghiglione et al., 2000). Genes involved in denitrification have been evidenced to be involved in (i) the fitness of bacteria during anaerobic growth and (ii) rhizospheric competence. Indeed, *nir* or *nor* mutants of *Nitrosomonas europaea* presented limited growth under denitrification conditions (Schmidt et al., 2004), whereas *nir* and *nor* mutants of *Paracoccus denitrificans* and *Rhodobacter sphaeroides* are unable to grow (De Boer et al., 1996; Bartnikas et al., 1997). Moreover, competition assays performed by Philippot et al. (1995) and Ghiglione et al. (2000, 2002) between wild type and *nir* or *nar* mutants of *P. fluorescens* YT101 on maize roots demonstrated that *nirS* and *narG* genes confer to the *P. fluorescens* YT101 strain a competitive advantage in rhizosphere colonization. In addition, Mirleau et al. (2001) demonstrated the role of nitrate reductase in the fitness of *P. fluorescens* C7R12 strain during *in vitro* and *in planta* assays (Mirleau et al., 2001). All these studies tend to evidence the role of denitrification genes (reductases) in rhizosphere competence.

Overall, to understand more deeply the advantage proffered by the reduction of TEAs, such as Fe(III) oxides in the rhizosphere for bacterial root colonization, a mutant of a model rhizobacterium affected in Fe(III) oxide reduction should be constructed. As done previously for denitrification, an experiment based on comparing the mutant's and the wild type's (WT) fitness in the rhizosphere could be an easy and accurate way to evidence the importance of TEAs reduction in the rhizosphere competence. Further investigations are needed to prove the competitive advantage of TEA reducers within a complex rhizospheric microbial community.

Anaerobic Respiration for Pollutant Removal

Anaerobic processes by microorganisms are involved in the degradation of a wide range of pollutants named "detoxification" (Harwood et al., 1998). Bacterial anaerobic respiration is capable of using selective-priority pollutants as TEAs and reducing them to non-toxic compounds through the respiratory electron transfer chain (Williams et al., 2013; Nanchaiah and Lens 2015). This process plays an important role in the removal

of contaminants and the remediation of soils. Several forms of bacterial anaerobic respiration and electron transfer related to the biotransformation of pollutants, including respiration with humic substances, sulfonates, halogenated chemicals, azo compounds, metallic elements, and non-metallic elements, were deeply reviewed by Hong and Gu (2009).

Non-metallic TEAs, such as As(V) and Se(IV), can be rendered unavailable from contaminated soils by anaerobic bacterial respiration. Indeed, As(V) is the major species of adsorbed arsenic found in contaminated-soils (Bissen and Frimmel, 2000). The reduction of this absorbed As(V) in more mobile and less adsorptive As(III) by dissimilar arsenate-reducing bacteria can facilitate As removal from soil's solid phase to an aqueous one and thus contribute to soil decontamination (Yamamura et al., 2007). In selenium-contaminated soils, highly toxic Se(V) can be reduced to less toxic Se(III) by certain selenium-reducing bacteria (Gerhardt et al., 1991). They so participate in the bioremediation of soil from Se(V). For certain bacteria, anaerobic reduction of toxic compounds does not always support growth in anaerobic conditions. In this case, anaerobic reduction of toxic oxyanions is coupled with another anaerobic respiration, such as Fe(III) and denitrification (Hunter and Kuykendall, 2007; Lee et al., 2012; Subedi et al., 2017).

Anaerobic Respiration for Bacterial Persistence in the Environment

The persistence of microorganisms relies on the ability to survive in the environment at low population density until conditions are more favorable (Kluepfel, 1993). Indeed, Fierer et al. (2012) suggested that adaptive mechanisms that facilitate better persistence for bacteria in adverse abiotic conditions are more important than competitive ones in shaping the microbial communities. In this respect, the ability of bacteria to grow in anaerobic conditions can be considered a persistence trait. Indeed, denitrification has been shown to contribute to bacterial persistence in extreme environments, such as the desert. Also, denitrifying enzymes are capable of tolerating extended periods of desiccation, allowing denitrifiers to better persist and to quickly respond to favorable conditions (Peterjohn, 1991). Regarding these results and the important differences of pO₂ occurring in the rhizospheres, it is of great interest to demonstrate in what extent the anaerobic respiration of available TEAs contributes to bacterial persistence in this particular biotope.

CONCLUDING REMARKS

The diversification of respiration in prokaryotes has made a major contribution to the ability of these microbes to colonize a wide range of environments from the oceans to the earth's crust and to adapt to changing environments, such as the rhizosphere. Improving our knowledge of bacterial respiratory flexibility in the rhizosphere is a major challenge, requiring a combination of a wide range of scientific expertise. Currently, the emergence of molecular approaches offers great potential for understanding respiratory processes at a rhizosphere scale. Approaches such

as TnSeq (Perry and Yost, 2014) and CRISPR cas9 (Hsu et al., 2014) can be used for characterizing respiratory pathways in rhizospheric model strains, whereas metagenomics (Delgado-Baquerizo et al., 2018) and RNAseq (Newman et al., 2016) approaches can be applied to rhizospheric bacterial communities. Gaining a greater understanding of how bacterial anaerobic respiration participates in root colonization and environmental adaptation and persistence is an exciting research challenge. Finally, to better understand anaerobic respiration in the rhizosphere, it is crucial to consider all the participants, such as fungi and archaea.

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AUTHOR CONTRIBUTIONS

SL and FH wrote the review, DA performed metatranscriptomic analyses and WA, TH, and XN reviewed and approved the final manuscript.

ACKNOWLEDGMENTS

We acknowledge funding from the French National Research Agency (ANR-18-CE32-0005, DIORE). The authors gratefully thank Hamid BAYA for the design of the figures.

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Denitrification is the anaerobic respiration most studied probably due to its widespread among microorganisms and notably soil bacteria and probably because anthropogenic reasons such as the extensive use of nitrogen-rich fertilizers (Sparacino-Watkins et al., 2013). This use generates an increase in denitrification activity and hence in greenhouse gas emissions. We propose to do a focus on denitrification, its genes, regulation and its roles in plant-bacterial interactions.

1.1 Focus on microbial denitrification

1.1.1 Nitrogen cycle: definition, imbalance and consequences

Nitrogen (N) is the fourth most abundant element in cellular biomass and our understanding of its cycle is in constant evolution (Stein & Klotz, 2016). The N cycle can be considered to start and finish with N_2 , is described as a network of oxidation-reduction reactions and is entirely driven by bacteria, archaea and fungi activities (Coskun et al., 2017).

The major steps of the N cycle are N_2 fixation, nitrification and denitrification. N_2 fixation consist in the reduction of N_2 to ammonium (NH_4^+) and is accomplished by bacteria or archaea that encode nitrogenase enzymes during free-living or symbiotic associations (Vitousek et al., 2013). Nitrogen fixation is extremely oxygen sensitive and requires microbial system adaptations, such as maximization of nitrogenase synthesis and/or specific plant structures, such as plant root nodules. Only bacteria and archaea have been described to perform this process. Nitrification consists in oxidation of NH_4^+ to nitrite (NO_2^-) *via* hydroxylamine (NH_2OH) and oxidation of nitrite to nitrate (NO_3^-). This process involves three different types of microorganisms: the ammonia-oxidizing bacteria and archaea (AOB and AOA), the nitrite-oxidizing bacteria (NOB) and the complete ammonia oxidizers (comammox) that perform both oxidative steps. Comammox microorganisms were only discovered in 2015 and only belong to the genus of *Nitrospira*. The reverse process, denitrification, is described as the anaerobic respiration of nitrogen compounds. It consists in the dissimilative reduction of nitrite to nitric oxide (NO), and nitrous oxide (N_2O) and finally back to N_2 . The reduction of nitrate to nitrite can be considered or not as a part of denitrification (Philippot, 2002). Denitrification decreases the leaching of nitrate to ground and water surfaces and is the main biological process responsible for the return of fixed nitrogen to the atmosphere (Fowler et al., 2013). Denitrification is performed by bacteria, archaea, and fungi.

Some other reactions also contribute significantly to terrestrial N cycling, such as the mineralization of organic N to NH_4^+ by fungi and heterotrophic bacteria (Bottomley et al., 2012) (**Figure 3**). Assimilatory (ANRA) and dissimilatory (DNRA) nitrate reduction to ammonium involve the use of nitrate as a terminal electron acceptor by bacteria and fungi. These microorganisms reduce NO_3^- to NH_4^+ *via* NO_2^- under anaerobic or low-oxygen conditions. The anaerobic ammonium oxidation (anammox) consists in the formation of N_2 from NO_2^- and NH_4^+ *via* the intermediates NO and hydrazine (N_2H_4). Anammox has been described to be performed only by bacteria. Finally, nitrogen could be

converted to NH_4^+ by the nitrate assimilation process that consists in the reduction of NO_3^- to NO_2^- and NH_4^+ (**Figure 3**).

The ongoing amplification of the global N cycle due to human activities has altered the biogeochemical processes of natural ecosystems (Bakken et al., 2012). Microbial activities regulate the retention or the loss of N from ecosystems and determine in which form it is lost (Hallin et al., 2018). Nitrous oxide is worthy of interest because it is a potent greenhouse gas with a global warming impact 300 times greater than that of carbon dioxide. It is also the major factor causing stratospheric ozone destruction. Terrestrial ecosystems are responsible for the majority of planetary N_2O emissions mainly because of the extensive use of fertilizers, dramatically increasing the amount of N present in agricultural soils. The NO_3^- and NH_4^+ provided by fertilizers and NH_4^+ formed during N_2 fixation can be incorporated by plants or used by microorganisms for their metabolism. Hence, plants and microorganisms compete for nitrogen, and this competition is particularly strong in the rhizosphere (Kuzyakov & Xu, 2013).

This significant imbalance has led to a number of negative environmental impacts in the atmosphere, in terrestrial ecosystems, and in freshwater and marine systems, such as acid rain, smog, eutrophication of coastal waters. Here, we are particularly interested in the greenhouse effect, climate change and stratospheric ozone depletion because these three effects are related to denitrification activity (Galloway et al., 2013).

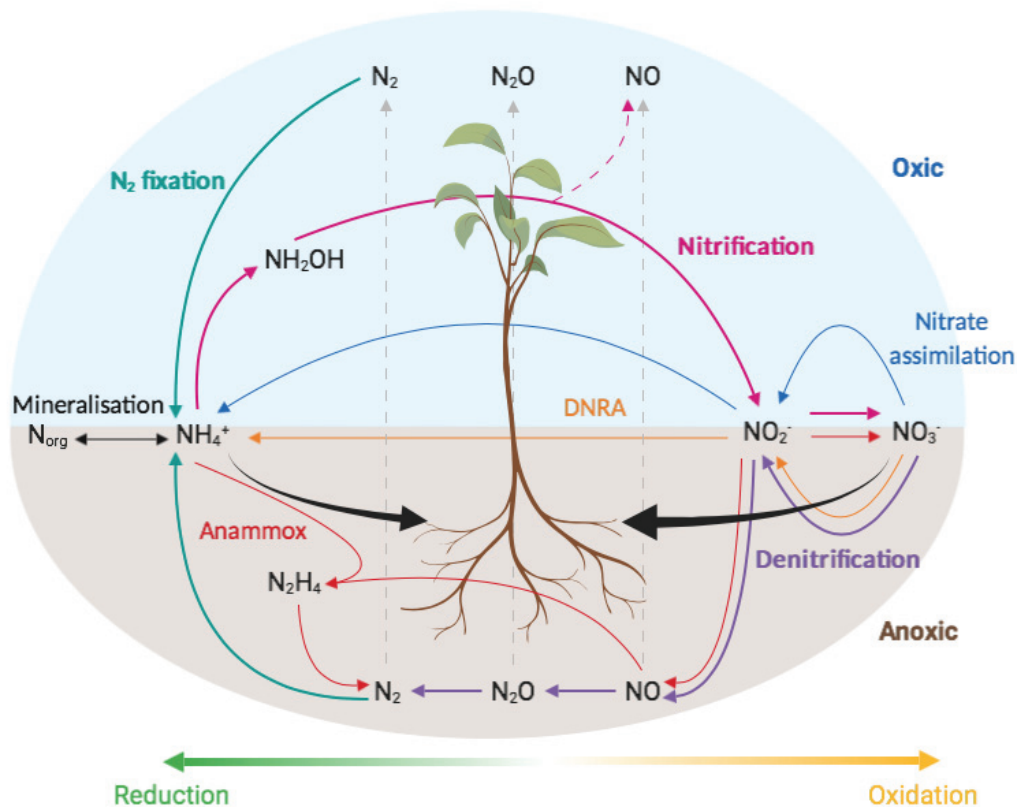


Figure 3. The soil nitrogen cycle driven by microbial transformations. A schematic overview of the microbial nitrogen transformations adapted from Coskun et al. (2017) and Hallin et al. (2018). Boldfaced colored lines indicate the major microbial pathways: N_2 fixation in green, nitrification in pink and denitrification in purple. Solid colored lines indicate the minor microbial pathways: DNRA in orange, anammox in red and nitrate assimilation in blue. Dotted lines indicate the gaseous nitrogen compounds released in the atmosphere. Boldfaced black arrows indicate nitrogen uptake by plant roots. Background colors represent oxic and anoxic conditions. The compounds are localized according to the oxidation state of N.

1.1.2 Denitrification: genes, regulators and role in plant interaction

Denitrification is a microbial process in which oxidized nitrogen compounds are used as alternative electron acceptors for energy production (Philippot, 2002). Denitrification is nearly an exclusively facultative trait (Zumft, 1997). This process is a part of the bioenergetic apparatus of the microbial cell. Nitrogen oxyanions serve as electron acceptors for the generation of the electrochemical gradient across the cytoplasmic membrane that leads to ATP production *via* an ATP-synthase (Zumft, 1997; Kern & Simon, 2009). Nitrogen is not incorporated into the biomass during dissimilatory denitrification. Instead, the end products of this dissimilatory process (NO_2^- , NO, N_2O , N_2) are exported from the cell (**Figure 4**). Exportation rates differ from species and depend of the efficiency of denitrification. *Diaphorobacter* sp., a denitrifying bacterium, showed nitrite accumulation in the media during the denitrification process at high amounts of nitrate, while *Paracoccus* sp. increases its denitrification efficiency under the same conditions (Chakravarthy et al., 2011).

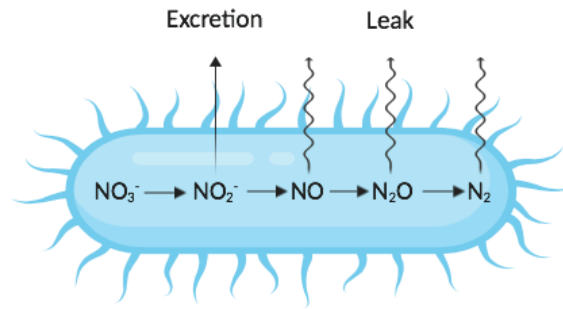


Figure 4. Nitrogen oxide flux during denitrification and nitrate assimilation (adapted from Sparacino 2014).

Denitrification is an inducible function which needs low oxygen pressure, nitrogen oxyanions and fresh carbon availability. However, aerobic denitrification has also been observed in bacteria, such as in *Paracoccus denitrificans* strains. Denitrification consists of four reaction steps by which nitrate is reduced into dinitrogen gas by four sequentially induced enzymes: nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase (for review see Zumft, 1997; Philippot, 2002). Here and for the rest of the manuscript, we will consider that nitrate reduction is the first step of denitrification.

1.1.2.1 Nitrate reduction

Four major groups of nitrate reductases have been described (eukNR, Nas, Nap, and Nar) and classified according to their physiological function, their cellular location (cytoplasmic, periplasmic, or membrane), their bioenergetic functioning and the structure of their molybdenum active site (Stolz & Basu, 2002). The respiratory nitrate reductase (Nar) and the periplasmic nitrate reductase (Nap) are dissimilative nitrate reductases. Dissimilatory nitrate reduction is an energy yielding process that generates ATP. Nar and Nap are involved in respiratory processes (denitrification and DNRA), detoxification, and redox regulation. By contrast, Nas is the assimilatory nitrate reductase that occurs during the nitrate assimilation process.

All nitrate reductases are molybdenumdependent enzymes because of their molybdenum active site, where the reduction of nitrate to nitrite occurs. This reduction requires two electrons and is coupled with the transfer of two protons. Nas is a cytoplasmic enzyme that requires ATP-dependent transporters to import nitrate into the cytoplasm. The two dissimilative nitrate reductases are membrane associated enzymes but differ by their location in the cell: membrane-bound for Nar and periplasmic-bound for Nap. The functioning of these reductases strongly differs because of this difference in cellular location. Nar contributes directly to energy conservation through the proton motive force by reducing nitrate in the cytoplasm and releasing protons into the periplasm. The

resulting electrochemical gradient can then be exploited to drive ATP generation *via* ATP synthase. By contrast, Nap does not directly contribute to the proton motive force because it reduces nitrate in the periplasm and no translocation of protons occurs (Moreno-Vivián et al., 1999; Kuypers et al., 2018). Respiration *via* Nap is predicted to generate less ATP than Nar (Kern & Simon, 2009; Sparacino-Watkins et al., 2013). The proton motive force across the inner membrane is produced by the coupled reduction of nitrogen compounds and oxidation of carbon compounds. In this case, carbon compounds act as electrons donors, whereas oxidized nitrogen compounds act as terminal electron acceptors. Even if Nap is less effective in generating an electrochemical gradient than Nar, the higher affinity for nitrate found in Nap can compensate this fact (Potter et al., 2001).

Multiple origins of nitrate reductases are suggested by Kuypers et al. (2018). Indeed, bacterial and archaeal Nas, Nap and Nar belong to the dimethyl sulfoxide reductase (DMSOR) family, whereas eukaryotic assimilatory nitrate reductases belong to the sulfite oxidase family. Interestingly, evidence for the coexistence of the three nitrate reductases has been provided for the *Paracoccus denitrificans* strain (Sears et al., 1997).

1.1.2.1.1 Nap, periplasmic nitrate reductase

Nap is widespread in denitrifying and non-denitrifying proteobacteria (Richardson, 2000). Unlike Nar and Nas, which have conserved functionality from species to species, Nap has been implicated in dissimilatory nitrate reduction or nitrate scavenging, suggesting that the presence of a *napA* in a genome does not necessarily confer respiratory nitrate reduction (Sparacino-Watkins et al., 2013).

Gene organization and composition of the *nap* operon vary significantly due to the high number of combinations of *nap* genes identified so far. In alpha-, beta-, and gamma-proteobacteria, the most common structure of the *nap* operon is *napEDABC*. The periplasmic nitrate reductase is a heterodimer encoded by the *napA* and *napB* genes and is located in the periplasm associated with membrane-anchored c-type cytochrome, encoded by *napC* (**Figure 5**). NapA is the catalytic subunit and NapB is a cytochrome. NapC mediates electron transfer between quinols and NapAB (Cartron et al., 2002). A *napD* gene encoding a protein, which catalyzes post-translational modification of NapA, is conserved in all bacteria studied to date. In addition to *napABDC*, *nap* operon may include one or more of the *napKEFGH* genes, but their occurrence and position in the operons differ between species. NapE and NapK are a small transmembrane protein with an unknown function. In *E. coli*, NapF interacts in the cytoplasm with NapA and acts as a chaperone by participating in the assembly of the iron-sulfur center of NapA (Nilavongse et al., 2006). In organisms lacking *napC*, NapG and NapH are predicted to transfer electrons from quinones directly to NapAB.

Nap can be controlled either by O₂ depletion and/or nitrate/nitrite availability or not according to studied species. In *Bradyrhizobium japonicum*, *nap* operon is induced under both O₂ depletion and nitrate/nitrite availability. By contrast, in *E. coli*, Nap is highly expressed at low nitrate concentrations under anoxic conditions. Interestingly in *Salmonella enterica*, a *napA* mutant exhibits a growth defect in the lumen of colon of mice suggesting that Nap is used to support growth under anoxic conditions and low nitrate concentrations (Lopez et al., 2015). In *P. denitrificans*, Nap is required for aerobic denitrification and is expressed under fully aerobic conditions (Ji et al., 2015). The high activity of NapAB allows denitrifying bacteria to preferentially use nitrate over O₂ to accept electrons under aerobic conditions (Ji et al., 2015).

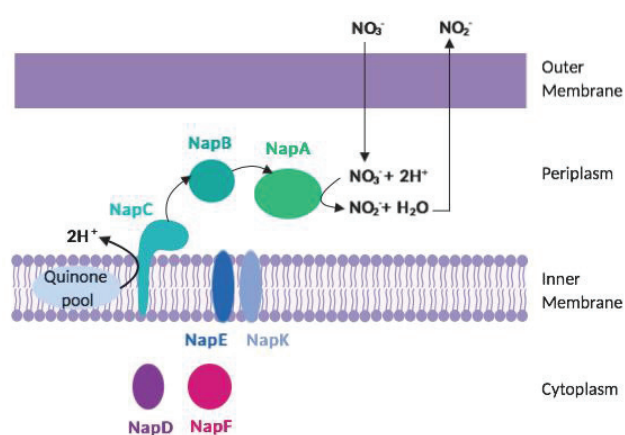


Figure 5. Localization and organization of Nap proteins in *Rhodobacter sphaeroides*. Bacterial membrane is represented in violet. The Nap system is represented in shades of green. The regulatory proteins NapDF and NapEK are represented in shades of pink and blue, respectively. The electron donor is the quinone pool symbolized by a blue oval. Adapted from Moreno-Vivián et al., 1999.

1.1.2.1.2 Nar, membrane bound nitrate reductase

Nar is found in both denitrifying and non-denitrifying-bacteria of all phyla (Van Spanning et al., 2005). Nar is induced during O₂ depletion and nitrate availability (Moreno-Vivián et al., 1999).

Nar proteins are encoded by genes of a *narGHJI* operon. By contrast with Nap, the organization of this operon is well conserved. Nar is a three-subunit enzyme composed of NarGHI. NarG is the catalytic subunit containing the molybdopterin cofactor. The cytoplasmic domain is constituted of NarGH while the membrane domain formed by NarI is required for the attachment of this complex to the cytoplasmic side of the inner membrane (Philippot, 2002). NarI participates directly to the proton motive force by oxidizing quinols and releasing two protons into the periplasm through NarG, *via* NarH, to reduce nitrate with the consumption of these two cytoplasmic protons (**Figure 10**). In addition, NarJ acts as a chaperone required for the maturation and membrane insertion of Nar (Blasco et al., 1990).

In *P. stutzeri* and *E. coli*, the expression of the *narGHJ* operon is under the regulation of the NarXL two-component system (Philippot, 2002). The NarX is a sensor protein encoded by the *narX* gene, which detects the presence of nitrate and nitrite in the periplasm, and the NarL is a DNA-binding regulator encoded by the *narL* gene, which regulates the target operon (Philippot, 2002). Interestingly, in *P. aeruginosa*, NarXL induces the expression of a small RNA Pail under denitrification conditions. Pail is required for efficient denitrification and controls the conversion of nitrite to nitric oxide (Tata et al., 2017). The Nar system is therefore involved in *P. aeruginosa* fitness. Furthermore, Fnr plays a central role in the expression of anaerobic metabolism genes in *E. coli* by binding to a consensus sequence upstream of the Fnr-regulated promoters (Moreno-Vivián et al., 1999). Fnr can act either as an activator or a repressor, depending on its location (Moreno-Vivián et al., 1999).

Because NarG is located in the cytoplasm, nitrate has to be transported into the cells before its cytoplasmic reduction (Moreno-Vivián et al., 1999). In denitrifying bacteria, NarK transmembrane transporters belonging to the major facilitator superfamily (MFS) of proteins are carried out for nitrate/nitrite transport (ref). NarK transporters are divided in two types of proteins: NarK1, involved in nitrate transport during assimilation pathway, and NarK2, involved in nitrate/nitrite antiporters during dissimilative nitrate reduction (Goddard et al., 2017). NarK and NarU of *E. coli* belong to this latter type of proteins (Zheng et al., 2013). In *E. coli*, NarK seems to act as a nitrate/nitrite antiporter, whereas NarU as a nitrate/cation symporter (**Figure 6**). In Pseudomonads, NarK seems to be a nitrate/nitrite antiporter (Vaccaro et al., 2015; Acuña et al., 2016). In *P. denitrificans*, two NarK proteins are present: NarK1, a nitrate/proton symporter, and NarK2, a nitrate/nitrite antiporter (Goddard et al., 2017). Recently, a new family of nitrate/nitrite transporters belonging to the MFS transporters and encoded by *narO* has been discovered in the extremely thermophilic bacterium *Thermus thermophilus* (Alvarez et al., 2019).

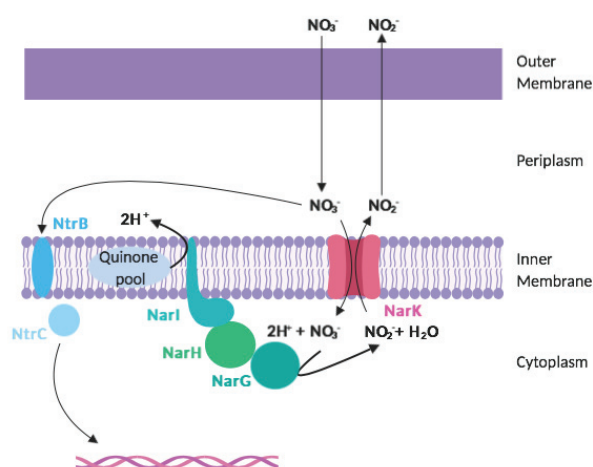


Figure 6. Localization and organization of Nar proteins in *E. coli*. Bacterial membrane is represented in violet. The Nar system is represented in shades of green. The two-component system NtrBC is represented in shades of blue. The electron donor is the quinone pool symbolized by a blue oval. The nitrate/nitrite antiporter NarK is symbolized in pink. Adapted from Moreno-Vivián et al., 1999.

NarG is essential for nitrate respiration. Indeed, in *P. fluorescens* C7R12, the role of NarG was evaluated by mutational and competitive assays. The growth of the *narG* mutant is defective and the wild type presents a selective advantage compared to the mutant *in vitro* (Mirleau et al., 2001). However, this selective advantage was not demonstrated in the bulk and the rhizosphere soil, indicating that this trait was not involved in rhizospheric competence (Mirleau et al., 2001). On the contrary, in another *P. fluorescens* strain, NarG confers a rhizospheric competence evaluated by measuring colonization and growth abilities of *narG* mutants (Ghiglione et al., 2000). In contrast, the growth of *S. enterica narG* mutants is not affected under anoxic conditions because of the functional redundancy of the nitrate reductase encoded by *napA* (Lopez et al., 2015).

1.1.2.1.3 Nas, nitrate assimilatory reductase

The nomenclature of the *nas* genes is confusing because different names have been given to homologous genes in different bacteria (Moreno-Vivián et al., 1999). However, the gene encoding the Nas system are normally clustered with nitrate/nitrite transport genes. In *Klebsiella pneumoniae*, *nasAC* encodes the nitrate reductase where NasA is the catalytic subunit and NasC is the diaphorase (Moreno-Vivián et al., 1999) (**Figure 7**). The nitrate/nitrite transport is encoded by *nasFED* genes and corresponds to an ABC transporter for nitrate and nitrite where NasF is a periplasmic binding protein, NasE a membrane protein and NasD an ATP-binding protein (Moreno-Vivián et al., 1999) (**Figure 11**). In *Synechococcus*, the NrtABC system is responsible for nitrate transport. NrtA is homologous to NapF, NrtB to NasE and NrtC to NasD (Moreno-Vivián et al., 1999). In *B. subtilis*, Nas is encoded by *nasBC* genes where NasC is the catalytic subunit and NasA is a nitrate transporter.

The assimilatory reduction of nitrate to ammonium is an energetically expensive process requiring eight electrons and active nitrate transport. Indeed, ABC (ATP-binding cassette)-type nitrate/nitrite transporters encoded by the *nas* cluster require energy (Luque-Almagro et al., 2011). Due to this energetic cost, the expression of the *nas* system is under a fine-tuned regulation based on nitrate/nitrite induction and ammonium repression. In bacteria, a two-component regulatory system NasST or NasR is in charge of this regulation (Luque-Almagro et al., 2011). NasS and NarR are two distinct nitrate/nitrite sensors and NasT is predicted to be a transcription anti-terminator (Luque-Almagro et al., 2011). The two-component system NtrBC is also involved in the control of the nitrate assimilation process in *Azospirillum brasilense* and *Rhizobium meliloti*. NtrB is a sensor kinase and NtrC acts as a transcriptional activator (Luque-Almagro et al., 2011) (**Figure 11**). Interestingly, Nas expression is repressed in ammonia-replete environments, such as fertilized soils, as a consequence of the requirement of ATP-dependent transporters (Kuypers et al., 2018).

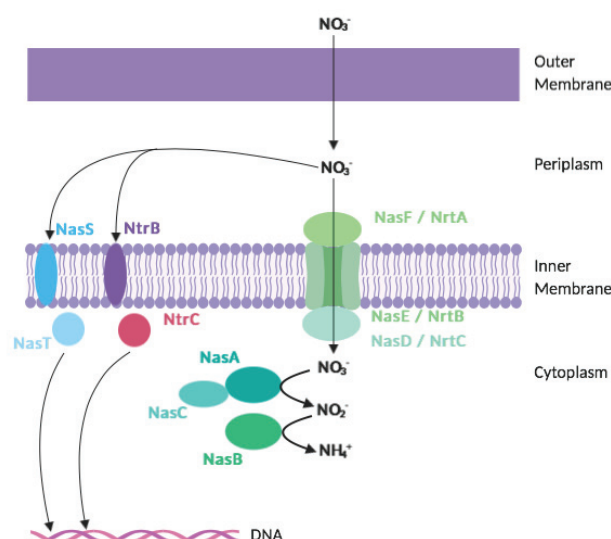


Figure 7. Localization and organization of Nas proteins in *Klebsiella oxytoca*. Bacterial membrane is represented in violet. The Nas system is represented in shades of green. The two-component systems NasST and NtrBC are represented in shades of blue and pink respectively. Adapted from Moreno-Vivián et al., 1999.

Some experiments with the nitrate reductase assimilation mutants demonstrated the role of Nas in growth on nitrate as a sole nitrogen source. In *P. syringae*, *nasB* mutant was unable to growth *in vitro* but was not affected when cultured in bean leaves (Parangan-Smith & Lindow, 2013). Moreover, in *Ralstonia solanacearum*, a *nasA* mutant exhibited reduced virulence and delayed stem colonization after soil soak inoculation of tomato plants. This delay can be explained by the requirement of NasA in normal extracellular polysaccharide (EPS) production that is necessary for root colonization. These results indicated that NasA promotes *R. solanacearum* growth, virulence, and competitive fitness in xylem tissue (Dalsing & Allen, 2014).

Table 3. Summary of nitrate reductase characteristics (From Moreno-Vivián et al., 1999)

| Characteristic | Nas | Nar | Nap |
|-------------------------------|---|---|---|
| Location | Cytoplasm | Membrane (cytoplasmic side) | Periplasm |
| Reaction catalyzed | $\text{NO}_3^- \rightarrow \text{NO}_2^-$ | $\text{NO}_3^- \rightarrow \text{NO}_2^-$ | $\text{NO}_3^- \rightarrow \text{NO}_2^-$ |
| Structural genes | <i>nasAC</i> | <i>narGHI</i> | <i>napAB</i> |
| Nitrate transport | Yes | Yes | No |
| Function | Biosynthesis | Denitrification | Denitrification |
| Regulation | | | |
| O_2 | No | Yes | Yes/No |
| NH_4^+ | Yes | No | No |
| $\text{NO}_3^-/\text{NO}_2^-$ | Yes | Yes | Yes/No |

1.1.2.2 Nitrite reduction

The reduction of nitrite to nitric oxide (NO) during denitrification pathways occurs in many environments in which nitrate is available and oxygen is limited (Kuypers et al., 2018). This reaction can be catalyzed by two evolutionarily unrelated enzymes: a copper-nitrate reductase (Cu-NIR) encoded by *nirK* and a haem-containing cd₁ nitrate reductase (cd₁-NIR) encoded by *nirS* (Philippot, 2002). These Nir are widespread among bacteria and archaea. NirS is found in Pseudomonads, such as *P. fluorescens*, *P. stutzeri* and *P. aeruginosa*. NirK is found in Rhizobia, such as *Sinorhizobium meliloti* and *Agrobacterium tumefaciens*. Interestingly, *Bradyrhizobium oligotrophicum* carries both nitrite reductases, NirK and NirS. A redundant role of *nirK* and *nirS* in denitrification is suggested for this strain by Sanchez and Minamisawa (2018). Both enzymes are periplasmic and catalyze the one electron reduction of nitrite to NO by receiving electrons from cytochrome c and/or copper protein (Van Spanning et al., 2005).

Commonly, *nirS* and *nirK* are used as gene markers to target denitrifiers in environmental studies. Indeed, *nirS* denitrifiers represent approximately 0.4% of total soil bacteria and *nirK* less than 0.2% (Kandeler et al., 2006). In microcosm experiments coupled to genomic analyses, Bremer et al. (2007) revealed the presence of *nirK*-type denitrifiers in the soil of eight plants. The authors suggested a role of denitrification in plant colonization. Bacteria harboring the *nirK* gene are less abundant than for those harboring *nirS*, but they are more taxonomically diverse (Wei et al., 2015). Moreover, the *nirS* denitrifiers are located preferentially in the rhizosphere, while *nirK* denitrifiers are more abundant in the bulk soil (Henry et al., 2004). The diversity and composition of *nirK* and *nirS* communities seem to be influenced by abiotic factors, such as temperature and pH (Bárta et al., 2010; Lee & Francis, 2017). Finally, these genes are present in many other microorganisms, including anaerobic ammonium-oxidizing bacteria, nitrite and methane-oxidizing bacteria, and ammonia-oxidizing bacteria and archaea, and so their role as markers is perhaps not the best choice as suggested by Kuypers (2018).

To determine the role of the nitrite reductase, the growth of the wild type (WT) and nitrite reductase mutant strains were compared under anoxic conditions (Philippot et al., 1995). In two *Pseudomonas* strains, the growth of the WT was significantly better than *nirS* mutants under anoxic conditions. Interestingly, *Pseudomonas* RTC01 survived better in non-sterile soil under anoxic conditions while *P. fluorescens* YT101 competed equally with the indigenous soil bacteria under oxic and anoxic conditions. Moreover, the presence of nitrite reductase conferred on both *Pseudomonas* strains a competitive advantage in an anaerobic environment and during rhizosphere colonization. This result demonstrated that denitrification can contribute to the persistence and distribution of bacteria in fluctuating soil environments (Philippot et al., 1995). In a different way, during pulmonary co-

infection with *Streptococcus parasanguinis*, the nitrite reductase of *P. aeruginosa* plays an important role for survival (Scofield & Wu, 2016).

1.1.2.3 NO reduction

NO plays an important role in plant immunity (Vaishnav et al., 2018), especially in plant stress tolerance mechanisms (Oz et al., 2015). Notably, NO induces a hypersensitive response during a pathogen attack (Delledonne et al., 1998). However, NO also participates in early basal signaling during plant-bacterial interactions. NO can act as a signaling molecule during bacterial-root association (Vaishnav 2018). In addition, nitrification and denitrification are a potent source of NO, and NO appears to be an important signal molecule for denitrifying bacteria to induce the expression of denitrification genes (Nadeem et al., 2013). However, at a high concentration, NO is toxic and highly reactive. So hypothetically, controlling the concentration of NO and thus the production and the use of NO by denitrifying organisms may be an advantage for plants and denitrifiers against competitors (Choi et al., 2006; Bakken et al., 2012). Interestingly, plant growth-promoting rhizobacteria (PGPR) including *Pseudomonas spp.*, *Azospirillum spp.* (Cutruzzolà, 1999) and phytopathogens including *R. solanacearum* (Dalsing et al., 2015) and *A. tumefaciens* (Baek & Shapleigh, 2005) harbor denitrification genes. In PGPR, NO can act as a biocontrol agent. For example, the antagonistic activity of a NO-overproducing mutant of a *P. fluorescens* strain against *R. solanacearum* infection of tomato plants was higher compared to the wild type (Wang et al., 2005). Moreover, PGPR protect roots against aggression from phytopathogens by enhancing biofilm formation *via* NO production (Compant et al., 2010). In addition, *Streptomyces sp.* enhances its antibacterial activity *via* the production of NO (Schimana et al., 2002) which allows the infestation of new ecological niches. Some pathogens using denitrification genes are able to detoxify the NO produced by plants, such as *R. solanacearum*. Using biochemical and genetic approaches, Dalsing et al. (2015) demonstrated that the initial growth of *R. solanacearum* in xylem is allowed by nitrate respiration, denitrification and NO detoxification, and the full virulence only required nitrite reduction and NO detoxification. The overproduction of endogenous NO seems to protect pathogens themselves from immunological oxidative stress (Shatalin et al., 2008). Taken together, these results suggest **that NO production by bacteria and plant-NO detoxification are essential to complete colonization and virulence in plant-pathogens.**

Rapid and effective reduction of NO by NO reductase into N₂O ensures that the free NO concentration in denitrifying organisms is kept under the toxic concentration (Van Spanning et al., 2005). Two types of nitric oxide reductase (Nor) have been identified in denitrifying bacteria: cNor in which electrons are received from c-type cytochromes and qNor in which electrons are received from

quinols. Both Nor are integral-membrane proteins and catalyze the two-electron reduction of two nitric oxide molecules to nitrous oxide (Van Spanning et al., 2005).

The nitric oxide reductase cNor has been described as a complex of two subunits: a membrane anchored c-type cytochrome and a cytochrome b, encoded respectively by *norC* and *norB* genes. The *norCB* genes are usually clustered with the *norQ* and *norD* genes corresponding to the four essential genes to express an active cNor (Philippot, 2002) (**Figure 8**). NorQ and NorD are maturation proteins essential for cNor activation (Jüngst & Zumft, 1992; Arai, Igarashi, & Kodama, 1995; Boer et al., 1996). In addition to this *norCBQD* operon, *norE* and *norF* are found in several bacteria, such as *P. denitrificans* and seem to be involved in maturation and/or stability of cNor activity (Boer et al., 1996) (**Figure 12**). The organization of the *nor* genes varies according to the presence/absence and the localization of *norEF*. The *nor* operon is regulated by NO and regulators from the CRP superfamily, such as Fnr (fumarate and nitrate reductase), Nnr (NO-sensitive regulator), and Dnr (dissimilatory nitrate respiration) (Philippot, 2002). Interestingly, the *norC* gene cannot be used as a gene marker to target denitrifiers in environmental studies instead of *nirK* and *nirS*, probably due to the lack of consensus sequences.

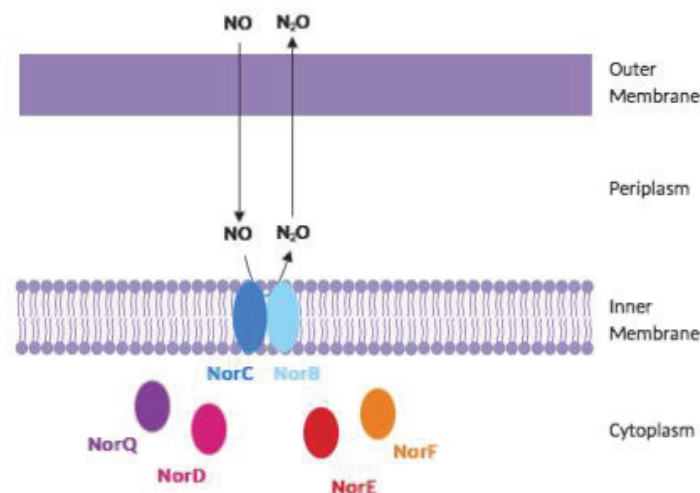


Figure 8. Localization and organization of Nor proteins in bacterial cells. Bacterial membrane is represented in violet. The NO reductase NorCB is in blue, the regulatory proteins norQD and norEF are in shades of pink and in shades of orange, respectively.

1.1.2.4 N₂O reduction

The ability to reduce N₂O is a taxonomically widespread trait, and not only denitrifying bacteria carry it. The N₂O reductase encoded by *nosZ* is the only known enzyme to reduce N₂O, a potent greenhouse gas. Interestingly, nearly 40% of denitrifying micro-organisms lack *nosZ* (Graf et al., 2014). The diversity of the *nosZ* gene can be divided into two distinct groups of N₂O reducers, clade I and II.

Clade I is dominated by denitrifiers whereas clade II is composed of denitrifiers and non-denitrifiers. These two clades are determined according to the phylogeny of the NosZ protein, the *nos* gene cluster organization, the NosZ translocation pathway and the frequencies of co-occurrence with other genes associated with denitrification or respiratory ammonification (for review see Hallin *et al.*, 2018).

Nos is a multicopper enzyme composed of two identical subunits. Typically, the *nos* gene operon is organized in three transcriptional units formed by the *nosZ* gene, the *nosR* gene and the *nosDFYL* genes (Philippot, 2002). The *nosZ* gene encodes the catalytic subunit consisting of the entry domain for electrons and the catalytic domain. The *nosR* gene encodes a regulatory protein. The *nosDFYL* operon encodes the NosD periplasmic protein, the NosF cytoplasmic protein containing both an ATP binding domain and an ABC transporter domain, the NosY hydrophobic protein, and the NosL Cu binding protein (Philippot, 2002). Additionally, in *S. meliloti*, NosX seems to be a maturation protein of NosZ activity. In *P. stutzeri*, NO and DnR are required for *nosZ* expression.

1.1.2.5 Known denitrification regulators

Globally, the oxygen depletion and the presence of nitrogen oxide, such as nitrate, nitrite and nitrous oxide induce *nir* and *nor* expression, whereas high oxygen concentrations repress their expression (Philippot, 2002; Van Spanning *et al.*, 2005; Bakken *et al.*, 2012). Moreover, pH negatively affects denitrification, with a reduction of N₂O emissions at a high pH level (Bakken *et al.*, 2012).

As denitrification is energetically unfavorable compared to O₂ respiration (Thauer *et al.*, 1989), this pathway must possess a fine-tuned regulation to minimize the electron flow to N-oxides in the presence of O₂ until denitrification becomes the only suitable respiration. In this way, denitrification could contribute to survival in natural environments subjected to rapid transitions from oxic to anoxic conditions. However, a minimal level of expression of denitrifying enzymes is necessary to sustain this rapid transition (Bergaust *et al.*, 2008). Coordinated expression and activity of the different reductases involved in denitrification are necessary to avoid toxic concentrations of nitrite and NO (Choi *et al.*, 2006).

The CRP superfamily of transcriptional activators is a global regulator of denitrification (for review see Gaimster *et al.*, 2018). Fnr, Nnr, Dnr, are members of this family and respond to oxygen and NO concentration to regulate denitrification targeted genes (Spiro, 2012). Global regulatory network patterns cannot be proposed since each strain presents its own network. For example, in *P. denitrificans*, the *nar* operon is under the control of NarR and FnrP which respond to nitrate availability and low O₂ concentration (**Figure 9**). The *nir* operon and the *nor* operon expressions are induced by

Nnr in the presence of NO and the absence of O₂. Finally, in the absence of O₂ and NO, FnrP induces *nosZ* expression (Spiro, 2012).

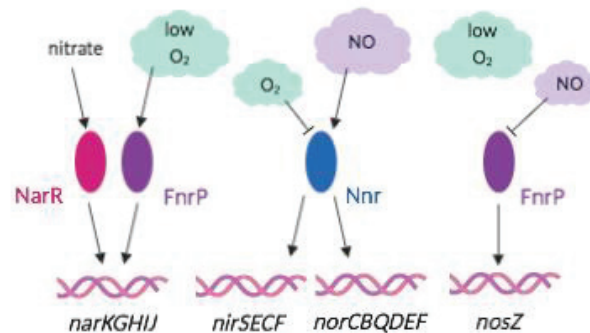


Figure 9. Denitrification regulatory network in *P. denitrificans*. Nitrate, O₂ and NO represent the regulatory signals, the regulatory proteins are symbolized by the ovals, and targeted structural genes are symbolized by the double helices. Arrows represent signaling events and gene regulation (Adapted from Spiro et al., 2012).

Non-coding RNAs (for review see Harfouche et al., 2015) may also play a role in denitrification regulation because they are specialized in the regulation of rapid environmental transitions, such as oxic/anoxic transitions. The repression of metabolic enzymes that have aerobic functions under low-oxygen conditions by non-coding RNA was already demonstrated in *Enterobacteria* (Boysen et al., 2010; Durand & Storz, 2010). Transcriptomic data are already available for several denitrifying bacteria that interact with plants, such as *S. meliloti* (Torres-Quesada et al., 2014) and *Agrobacterium fabrum* C58 (Dequivre et al., 2015). **Further studies are needed to identify non-coding RNA involved in denitrification regulation and to understand their global involvement in denitrification pathway regulation.**

1.1.2.6 Concluding remarks

Denitrification is widespread among soil microorganisms. The nitrate reduction is catalyzed by 3 different enzymes: Nap, Nar and Nas. The respiratory nitrate reduction that occurs during denitrification is only catalyzed by Nap and Nar. Genetic organization of the *nar* operon is well conserved among bacteria whereas that of the *nap* operon is not. The nitrite reduction is catalyzed by 2 different enzymes: NirK and NirS. Both nitrate and nitrite reductases have been reported to be involved in bacterial fitness under anoxic conditions in certain model strains. The production of NO by Nir and the detoxification of NO by NorB have been reported to play a role in plant-bacterial interactions and virulence. The reduction of N₂O by NosZ allows the return of nitrogen into the atmosphere.

Incredible efforts have been undertaken to understand global denitrification activities and denitrifying communities (Domeignoz-Horta et al., 2018; Pajares et al., 2018; Putz et al., 2018) in order to reduce N₂O emission. We think that elucidating the mechanism of denitrification regulation on model strains would also help to propose strategies to reduce N₂O emission and to better understand the role of denitrification in plant colonization. **We propose to decipher denitrification pathways and regulation in *Agrobacterium fabrum* C58, a well-known rhizobacterium colonizing efficiently plant host.** Learning about saprophytic and pathogenic lifestyles of *A. fabrum* C58 will participate to better understand the role and regulation of denitrification in niche colonization.

2 *Agrobacterium* spp.

2.1 Genomic species complex concept

In asexually reproducing organisms, species are defined upon similarities of their members. In bacteria, strains were first classified in different species according to phenotypic traits (Sneath & Sokal, 1973). The current definition of genomic species belongs to the discovery of similarities and discontinuities at the genomic level. Indeed, the genomic DNA hybridization ratio for pairwise comparisons fixed at 70% is in agreement with the previous phenotype-based distinction of species. Two strains belong to the same species if they reach this limit of 70% of similarity.

Lassalle et al. (2011) propose a new definition of genomic species more adapted to *Agrobacterium tumefaciens*. In their view, “a genomic species is likely descending from a single ancestor that speciated a long time ago consecutively to adaptations to a novel ecological niche”. This adaptation leads to the presence of species-specific genes in genomes of all members of a given species but not in closely related species. For them, “species-specific genes inherited from the ancestor may still be responsible for the adaptation of present species members to a species-specific ecological niche”. Currently, *A. tumefaciens* is a complex of 11 distinct genomic species, named genomovar G1 to G9, G11 and G13 (**Figure 10**) (Costechareyre et al., 2010).

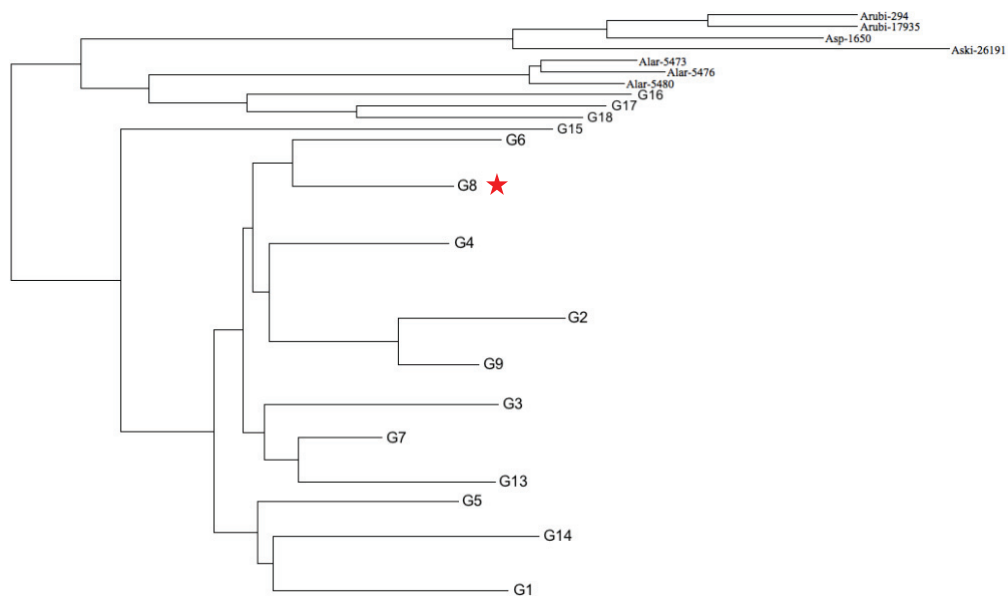


Figure 10. Phylogenetic tree of *Agrobacterium tumefaciens*. This tree has been constructed with the *recA* gene. The grey color represents *A. tumefaciens* genomic species. The red star represents the genomic species to which the model strain *A. fabrum* C58 belongs.

Agrobacteria belong to *Alphaproteobacteria* are found in soils in the free living form as well as in association with plants in the rhizosphere (Chèneby et al., 2004; Haichar et al., 2008). *Agrobacteria* acquire its virulence from the Ti (Tumor inducing) plasmid. When it is present, bacteria can switch from a non-pathogenic lifestyle to a pathogenic one and induces tumors on plants called crown gall disease. Different types of pTi are found in *A. tumefaciens* and are classified according to the type of opine produced by the tumor, such as octopine or nopaline (Beck von Bodman et al., 1992). Surprisingly, *Agrobacteria* isolated from soils and rhizosphere are most often avirulent (Krimi et al., 2002). Interestingly, several strains and genomic species are found in the same soil samples (Vogel et al., 2003; Costechareyre et al., 2010). According to the competitive exclusion principle (Hardin, 1960), this co-occurrence of *Agrobacterium* strains may be allowed by species-specific genes leading to the adaptation of partly different ecological niches. Several traits are proposed for rhizosphere competence, such as metabolic versatility, anaerobic respiration flexibility, siderophore production, and antimicrobial compound resistance (for review see Leonard et al., 2017; Dessaux & Faure, 2018; Meyer et al., 2019).

A. fabrum C58 is the model strain of genomovar G8 and is completely sequenced. *A. fabrum* C58 carries 4 replicons: a circular chromosome of 2,8 Mb, a linear chromosome of 2 Mb, a megaplasmid of 540 kb named pAT and an accessory plasmid pTi of 210 kb (Goodner et al., 2001; Wood et al., 2001a). This bacterium was used as model strain during this thesis.

2.2 *Agrobacterium* lifestyles

Recently, Dessaux and Faure (2018), Barton *et al.* (2018) and Meyer *et al.* (2019) reviewed the abilities of *Agrobacterium* to survive and compete in soil and plant environments. These reviews highlight the fact that soil colonization by *Agrobacterium* is much less known than its abilities to construct a plant-niche, survive in the tumor and exploit plant resources. **Highlighting *Agrobacterium* traits that favor the adaptation and colonization of soil and rhizosphere environments will continue to be a great challenge for future studies.**

2.2.1 Soil and rhizosphere niche colonization

Agrobacterium are soil and rhizosphere-adapted bacteria, colonizing these environments at 10^3 to 10^7 CFU/g (Bouzar & Moore, 1987; Bouzar *et al.*, 1993; Krimi *et al.*, 2002) (**Figure 15**). *Agrobacteria* exhibit several traits to explore and colonize a wide range of environments (Dessaux & Faure, 2018). Indeed, *agrobacteria* are able to uptake and synthesize osmo-protectants in order to develop an osmo-resistance (Dessaux & Faure, 2018). Motility and chemotaxis *via* flagellar locomotion are also important traits for the early steps of host colonization in *A. tumefaciens* (Pérez Hernández *et al.*, 1999; Merritt *et al.*, 2007). Once *Agrobacteria* reach the roots, they will form biofilms. Surface adherence affords microorganisms a number of survival benefits, such as protection from desiccation, antimicrobial compounds and host defense responses (Davey & O'toole, 2000; Lewis, 2005). *A. tumefaciens* forms dense, complex biofilms on both abiotic and plant surfaces that seem distinct from other plant-colonizing soil bacteria (Auerbach *et al.*, 2000; Ramey *et al.*, 2004). *A. tumefaciens* persists in a complex terrestrial environment composed of living plant tissue, plant matter and inorganic soil material probably through specific biofilm formation. Ramey *et al.* (2004) identified through mutant analysis SinR, a biofilm regulator member of the Fnr family. Interestingly, they proposed a model in which SinR is activated by FnrN in response to oxygen limitation. **This result suggests a link between oxygen level and biofilm formation in *A. tumefaciens*.** Moreover, anaerobic respiration could be involved in soil colonization by *A. tumefaciens* species. Remarkably, genes encoding denitrification pathways are unequally spread among the genomic species of *A. tumefaciens* complex (Lassalle *et al.*, 2017). **This observation suggests that anaerobic respiration abilities could drive the soil distribution of *A. tumefaciens* genomic species.**

Using plant compounds for its own growth is also a strategy for colonizing and competing with other microorganisms. *Agrobacteria* have developed a wide metabolic capacity. They can survive on humic acids (Süle, 1978), a potent terminal electron acceptor. They can also degrade a large range of monosaccharides, polyols, and sugar derivatives often of plant origin (Dessaux & Faure, 2018). Sugars of the raffinose family oligosaccharides (RFO) are produced by plants during the early steps of

germination. Interestingly, *A. fabrum* C58 is able to uptake RFO to preferentially colonize seeds (Meyer et al., 2018). The periplasmic binding protein MelB is conserved among *Agrobacteria* and *Rhizobiaceae* and is suggested to be essential for plant colonization (Meyer et al., 2018). **The ability of *A. fabrum* C58 to use a wide range of organic compounds may allow it also to use them as terminal electron acceptors for anaerobic respiration for niche colonization.**

2.2.2 Tumor environment

The pathogen *A. tumefaciens* is able to form a plant-tumor called crown gall when harboring the Ti plasmid (pTi). Indeed, this plasmid contains a DNA region transferable to plant cells, called T-DNA. Once the T-DNA is integrated into the host nuclear genome, plant hormones auxin and cytokinin are produced and provoke the development of the tumor. Interestingly, this novel environment is colonized by *A. tumefaciens*, therefore becoming a quasi-specific niche or at least a more private habitat (Gohlke & Deeken, 2014; Dessaux & Faure, 2018). Moreover, T-DNA encodes plant metabolites known as opines. Cleverly, *A. tumefaciens* uses these opines as nutrients through genes encoded by the pTi and so acquires a selective growth advantage over the other rhizosphere microorganisms (Oger et al., 1997; Mondy et al., 2014). In this niche, *A. fabrum* C58 can also use other metabolites specifically enriched in this environment as nutrients, such as γ -aminobutyric acid (GABA), pyruvate and γ -hydroxybutyric acid (GHB) (Gonzalez-Mula et al., 2019).

Two opposite views are found in the literature about the *Agrobacteria* tumor life. Indeed, the tumor environment constructed by *Agrobacteria* can be perceived as a hostile environment or as an opportunistic one. On one hand, Dessaux & Faure (2018) consider that the construction and the colonization of the tumor-niche provide *A. tumefaciens* with a prolific environment in which resources increase while plant defense response and microbial competition decrease. On the other hand, Meyer et al. (2019) introduce the mechanisms that *Agrobacteria* must develop to survive in this hostile tumor environment. One way or the other, it is clear that a **tumor is a constructed niche where *Agrobacteria* proliferate through metabolic adaptation, such as opine degradation, osmoprotection establishment, oxidative stress resistance and probably anaerobic respiration activation (Figure 11).** Indeed, as tumor niche is a rich environment with carbon and nitrogen sources (Gonzalez-Mula et al., 2019) and that oxygen can be rapidly a limiting factor (Deeken et al., 2006), **denitrification and other anaerobic respirations may occur.**

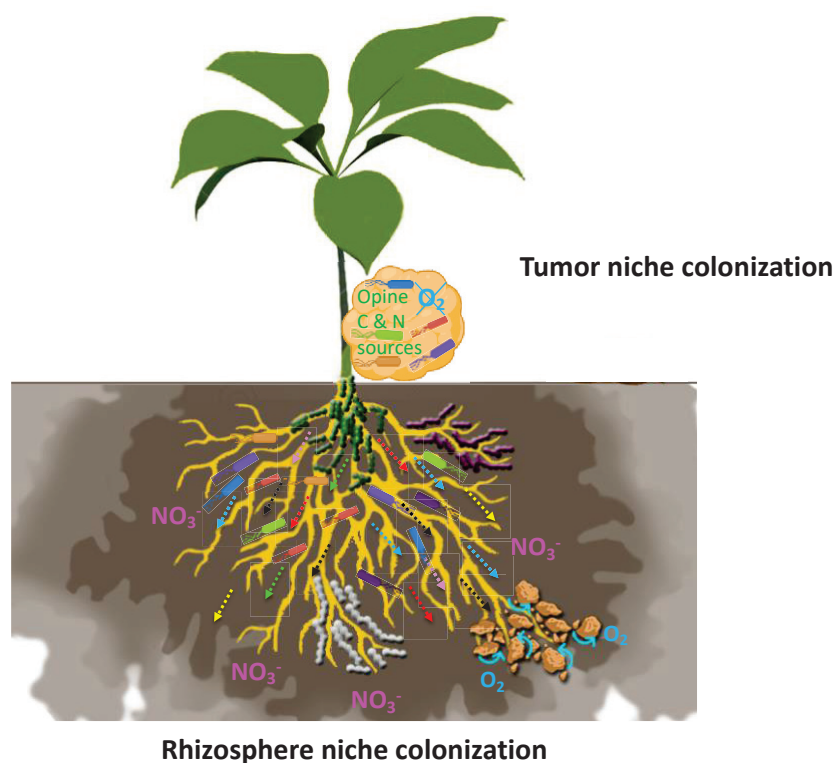


Figure 11. Rhizosphere and tumor niche colonization by *Agrobacterium fabrum* C58. Diversity of bacteria and *Agrobacterium* is represented by multiple colors (red, orange, blue, purple, green) and shapes. Tumor niche is represented in yellow. O_2 and NO_3^- are represented in blue and pink, respectively.

2.3 Denitrification in *A. fabrum* C58

A. fabrum C58 is a partial denitrifier that lacks the NosZ reductase and harbors a denitrification gene cluster of approximately 60 kb located on the linear chromosome (Goodner et al., 2001; Wood et al., 2001b). The accurate re-annotation performed by Baek & Shapleigh (2005) revealed the presence of the copper-containing Nir protein encoded by the *nirK* gene and the nitric oxide reductase Nor encoded by *norCBQD* operon. **The role of NapA as a nitrate reductase was never investigated even though Gonzalez Mula et al. (2018) has suggested its involvement in *A. fabrum* C58 denitrification.**

During the rapid transition from an oxic to an anoxic environment, denitrification can contribute to survival only if this pathway's regulation is fine-tuned. In *A. fabrum* C58, *nirK* and *norB* expression is regulated by the presence of nitrate, nitrite and NO. Bergaust et al. (2008) demonstrated that it was not always easy for the cells to make a successful shift between respiratory pathways. The optimal conditions that facilitate the successful transition from oxic to anoxic conditions and successful denitrification are 1-2 μM of O_2 , 2 mM of nitrate or nitrite and a neutral pH (Bergaust et al., 2008). All difficulties come from the production of energy required for denitrification gene expression. Indeed, O_2 is the better choice of energy required for early denitrification gene expression (Bergaust et al.,

2008). When *A. fabrum* C58 is confronted with a rapid transition from oxic to anoxic conditions, it appears unable to perform a balanced expression of *nirK* and *nor* in the presence of nitrate, resulting in extremely high emissions of NO (Bergaust et al., 2008; Bakken et al., 2012). In contrast, this transition is easier on nitrite, most likely because NO is produced from Nir and from dismutation of nitrite, and this NO slows O₂ respiration leading to a deceleration of the transition and allowing denitrification gene expression (Bergaust et al., 2008). In the meantime, NO is required for maximal *nirK* and *nor* expression (Baek & Shapleigh, 2005; Bergaust et al., 2008). However, *nirK* and *nor* are not under identical transcriptional regulator control. Indeed, *nirK* expression depends on at least three transcriptional activators, NnrR, FnrN and ActR, whereas *nor* expression depends on NnrR and FnrR to prevent NO accumulation (Baek et al., 2008). ActRS is a two-component system where ActR is the regulator and ActS is the sensor. Indeed, the activation of *nnrR* expression by FnrN and the increase of phosphorylated ActR due to changes in the redox status of the electron transport chain are concomitant with the decrease of O₂. The production of NO in presence of nitrate and nitrite will also activate NnrR, leading to an autoregulation of this mechanism and to the increase of *nirK* expression. This fine-tuned regulation ensures that *A. fabrum* C58 can switch more effectively from oxic to anoxic life.

The expression of *A. fabrum* C58 denitrification genes has been evaluated in roots, leaves and tumors using mutant assays and transcriptomic approaches (Baek & Shapleigh, 2005; González-Mula et al., 2018). The authors clearly demonstrated the induction of *nor* gene expression probably activated by plant-derivative NO. No data indicates whether the conditions are favorable for denitrification establishment and hence for the induction of nitrate and nitrite reductase gene expression. Regarding experimental procedures, no conclusion on the involvement of *A. fabrum* C58 denitrification in fitness and plant colonization can be provided. In this thesis, we propose **to study nitrate reductase role in *A. fabrum* C58 fitness and plant colonization.**

2.4 Concluding remarks

Agrobacterium tumefaciens is a genomic species complex found in the soil, the rhizosphere and in the plant tumor environment. *A. fabrum* C58 belongs to the genomic species G8 and is the model strain used in this thesis. *A. fabrum* C58 is a partial denitrifier colonizing efficiently plant rhizosphere and tumor and able to use a wide range of carbon and nitrogen substrates. Great advances in the characterization of *A. fabrum* C58 denitrification have been undertaken, however, some gaps do still exist. For example, no data is available on which reductase catalyzes the respiratory reduction of nitrate to nitrite and on its role in *A. fabrum* C58 fitness and root colonization. In addition, learning about other anaerobic respirations that may occur in the rhizosphere in the absence of denitrification

is needed to understand *A. fabrum* C58 metabolic diversity and flexibility. **Filling the information gaps concerning anaerobic respiration abilities and rhizosphere colonization are a great challenge.**

3 Hypothesis and objectives

Anaerobic respiration may be an essential trait in lifestyle, environment colonization and survival. Until now, the only confirmed anaerobic respiration in *Agrobacterium spp.* is denitrification. Interestingly, this pathway is unequally widespread among Agrobacteria. In this context, the hypothesis of my thesis is that anaerobic respiration and notably denitrification could explain the coexistence of Agrobacteria and their distribution in specific niches in the rhizosphere. In order to test this hypothesis, we need first to better characterize denitrification respiration and to evidence whether other anaerobic respirations may occur in the rhizosphere. We proposed in this thesis to use first *A. fabrum* C58 as model strain (member of *Agrobacterium tumefaciens* species complex). The objectives of this thesis were to (1) complete the characterization of denitrification pathway in *A. fabrum* C58, (2) explore denitrification role in *A. fabrum* C58 fitness and root colonization and (3) discover new anaerobic respirations that may occur in plant rhizosphere (**Figure 12**).

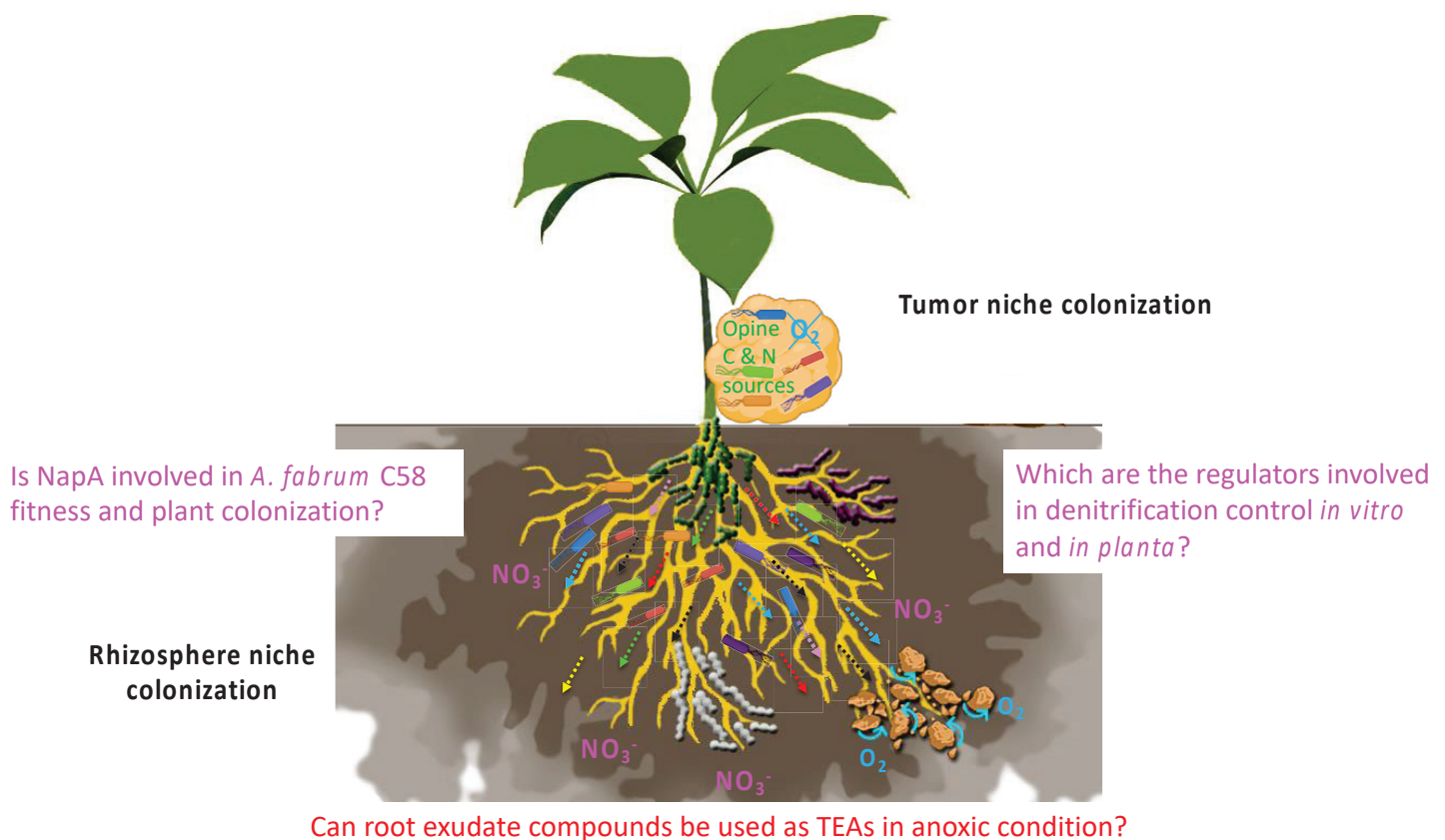


Figure 12. Hypothesis and objectives

- **Denitrification genes in *A. fabrum* C58 *in vitro***

To complete denitrification pathway in *A. fabrum* C58 and identify all the genes and regulators involved in the denitrification function, we adopted two strategies:

Firstly, a two-step method based on the identification of candidate genes and on the validation of a fitness trait by confronting strains carrying allelic variation to (1) identify the nitrate reductase involved in the first step of denitrification and (2) validate the role of small RNA (NopR) in denitrification control. To do so, we constructed $\Delta napA$ deficient strain, strain overexpressing NopR and another one reduced in NopR expression. Growth and capacity to produce N_2O under anoxic conditions were evaluated.

Secondly, to identify all the genes involved in denitrification, we constructed a mutant transposon library of *A. fabrum* C58 and tested its growth under denitrification conditions *in vitro* in the presence of either nitrate or nitrite.

- **Role of *A. fabrum* C58 denitrifying genes in maize root colonization**

To date, Transposon-sequencing (Tn-Seq) is one of the most powerful method to determine genes required for bacterial growth in the presence of their host (Royet et al., 2018). To determine denitrifying genes involved in root colonization under anaerobic conditions, we used the library constructed in *A. fabrum* C58 and inoculated it on maize plants. Then, these plants were grown on fertile-ground and cultured under flooded conditions to mime anoxic conditions. Sequencing the recovered *A. fabrum* C58 cells is under progress and will evidence the genes involved in this niche colonization in anoxia.

- **Discovery of new anaerobic respiration pathways in *A. fabrum* C58**

As *A. fabrum* C58 strain deficient in nitrate reductase was able to colonize maize roots under anoxic conditions, we hypothesized that primary metabolites exuded by maize plant can serve as terminal electron acceptors (TEAs). To discover new anaerobic respirations, we set-up a method to screen potential TEAs used by *A. fabrum* C58. TEAs were selected among primary metabolites exuded by maize plants and identified by HPLC.

Chapter 1

NapA is a nitrate reductase involved in *Agrobacterium fabrum* C58 denitrification

Preamble

Denitrification process has been well studied in *Agrobacterium fabrum* C58 (Baek & Shapleigh, 2005; Baek et al., 2008; Bergaust et al., 2008). Nitrite reductase NirK and nitric oxide reductase NorC have been characterized by mutant analysis (Baek & Shapleigh, 2005). Expression data are also available for *nirK* and *norCB* gene of *A. fabrum* C58 in roots and leaves of *Arabidopsis thaliana* (Baek & Shapleigh, 2005) and in tumor (González-Mula et al., 2018). Finally, transcriptional regulators FnrR, NnrR and ActRS control directly or indirectly *nirK* and *norCB* expression in *A. fabrum* C58 (Baek et al., 2008). However, no direct demonstration is available on the gene involved in nitrate reduction and its role on *A. fabrum* C58 fitness and root colonization. In addition, non-coding RNA are known to act during environmental rapid transition, and we assume that they can play a role in denitrification regulation. In this context, we will **i) characterize the gene encoding the nitrate reductase and its role in fitness and root colonization under anoxic conditions and ii) evaluate the potent role of a non-coding RNA evidenced previously by Dequivre et al. (2015).**

For this purpose, we constructed C58 Δ *napA*, a NapA deficient strain. The growth and the denitrification activity were evaluated for this strain and compared to the C58 wild type (WT). NapA is definitively the nitrate reductase involved in nitrate reduction to nitrite in *A. fabrum* C58. We performed competitions assays to determine the fitness of C58 Δ *napA* compared to C58 WT during *in vitro* and *in planta* assays. NapA is involved in C58 fitness *in vitro* because NapA-deficient strain was unable to growth and to denitrify compared to the WT. However, NapA is not essential for maize root colonization under anoxic conditions.

The role of the non-coding RNA called NopR (denitrification regulation small RNA) in denitrification control was tested by mutant construction and Northern Blot analysis. NopR is a cis-encoded antisense non-coding RNA that is 80 nt long, located on the opposite strand of the *norQD* genes, and precisely overlap the 3'-UTR of *norQ* mRNA by about 30 pb. NopR is expressed under anoxic conditions and is conserved among *Agrobacterium tumefaciens* genomic species harbouring *nor* operon. We constructed two strains modified in NopR level, one overexpressing it called NopR⁺, other silencing it called NopR⁻. The growth and the denitrification rate of these two strains were evaluated and compared to the C58 WT. Northern Blot were performed to propose a model for NopR regulation of *nor* operon. NopR seems to positively affect *norQ* mRNA by increasing its stability and hence increasing N₂O emission *via* NorC activation, as NorQ is a maturation protein that activates NorCB.

Results of this work are presented in a manuscript in revision in **Environmental Microbiology journal**.

NapA is a nitrate reductase involved in *Agrobacterium fabrum* C58 denitrification

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Running title: Nitrate reductase in C58: identification and role

Summary

Agrobacterium fabrum C58 is a plant-associated bacterium that is capable of anaerobic denitrification. By constructing a mutant that was defective in the first step, we demonstrated that the reduction of nitrate to nitrite is catalysed by the NapA reductase, which is located in the cluster 2 encoding the NirK and NorC reductases. The ability to denitrify is not found in all species of *Agrobacterium*, as demonstrated by measurement of N₂O emission and bacterial growth of 11 strains representative of 10 species of the biovar 1 species complex (e.g. *Agrobacterium tumefaciens* complex). Only species harbouring cluster 2 were able to transform nitrate to N₂O, indicating that denitrification is a species-specific pathway in *Agrobacterium* spp. The role of the NapA reductase in the fitness and maize root colonization behaviour of *A. fabrum* C58 was studied by performing competition assays *in vitro* and *in planta* as well between the wild-type and mutant strains. Our results demonstrate a positive role for NapA in the C58 fitness *in vitro* and suggest that the C58 Δ *napA* mutant uses components exuded by plant roots to respire anaerobically. We also demonstrated, for the first time, the involvement of a non-coding RNA, located in the *norCBQD* gene cluster, in the regulation of denitrification in *A. fabrum* C58.

1 Introduction

The diversification of respiration in prokaryotes has been a major contributor to the ability of these microbes to colonize a wide range of environments from the oceans to the Earth's crust (Richardson, 2000) and to adapt to changing environments, such as the rhizosphere (Haichar et al., 2014). Among the mechanisms of anaerobic respiration, the ability to denitrify has been shown to be advantageous to bacteria in the rhizosphere and was suggested to be a rhizospheric competence trait (Lecomte et al., 2018). Denitrification is a key function for the biochemical cycling of nitrogen. This process is a major source of atmospheric nitrous oxide (N₂O) and contributes to the emission of nitric oxide (NO). Emission of NO is undesirable because NO is toxic, and N₂O is a potent greenhouse gas (Houghton et al., 2001) and dominant ozone-depleting substance (Ravishankara et al. 2009). The denitrification process sustains respiratory growth under oxygen limitation *via* the dissimilatory reduction of nitrate to gaseous end products (Zumft, 1997). Denitrification consists of four successive steps: the first step (NO₃⁻ → NO₂⁻) is catalysed by the *narG* or *napA* gene, encoding nitrate reductase; the second step (NO₂⁻ → NO) is catalysed by the *nirK* or *nirS* gene, encoding two different types of nitrite reductase; the third step, leading to N₂O formation (NO → N₂O), is mediated by the *cnorB* or *qnorB* gene, encoding nitric oxide reductase; and the final step, reduction of N₂O (N₂O → N₂) is mediated by the *nosZ* gene, encoding nitrous oxide reductase (this step constitutes the only known microbial process that can reduce N₂O to N₂ in the biosphere) (Zumft 1997; Jones et al., 2013; Hallin et al., 2018). Unfortunately,

nearly one-third of *nirS*- or *nirK*-containing denitrifiers lack the *nosZ* gene (Bakken et al., 2012; Philippot et al., 2011), and therefore, these *nosZ*-lacking microbes do not have the genetic capacity to reduce N_2O and hence participate in N_2O emission and N loss from soils. An understanding of the physiology and regulatory biology of key N_2O -producing microorganisms may lead to the development of strategies to eliminate $\text{NO}/\text{N}_2\text{O}$ production and/or promote $\text{NO}/\text{N}_2\text{O}$ consumption at the microbial community level.

To date, different regulators have been reported for their involvement in the regulation of denitrification, for example, the Crp-Fnr superfamily regulators, such as Fnr (fumarate and nitrate reductase) and NnrR (response to NO in denitrification) (Zumft 2002; Körner et al., 2003). Fnr is a transcriptional regulator that controls the expression of the genes required for anaerobic metabolism in response to O_2 availability (Zumft 2002). NnrR is a protein that controls the free NO concentration by regulating the expression of NO-producing (NIR) and NO-consuming (NOR) enzymes (Bartnikas et al., 1997; Tosques et al., 1996). Regarding NO toxicity, regulation of NO production and consumption is a critical step in the denitrification process, especially for rhizospheric bacteria that are subjected to different environmental changes, such as rapid transition from oxic to anoxic conditions.

Among *nosZ*-lacking bacteria, *Agrobacterium fabrum* C58 is a facultative denitrifying bacterium that belongs to Alphaproteobacteria and is found in soils in free living form as well as in association with plants in the rhizosphere (Chèneby et al., 2004; Haichar et al., 2008). The *A. fabrum* C58 genome carries on its linear chromosome a gene cluster of approximately 60 kb that is involved in denitrification (Goodner et al., 2001; Wood et al., 2001). The accurate re-annotation performed by Baek & Shapleigh (2005) revealed the presence of the copper-containing Nir protein (encoded by the *nirK* gene) that is involved in nitrite reduction and the *nor* gene cluster, encoded by *norCBQD*, that is involved in nitric oxide reduction (Fig. 1a). Annotation of the *A. fabrum* C58 genome revealed the presence of two putative nitrate reductases: NasA and NapA. NasA is the assimilatory nitrate reductase that occurs in nitrogen assimilation pathway, whereas NapA is the respiratory nitrate reductase that occurs in denitrification pathway. However, even though nitrate reduction is the most energetically favourable step for denitrifiers, no evidence is available regarding which of these two reductases catalyses the respiratory reduction of nitrate to nitrite in *A. fabrum* C58. In this species, it is well known that NnrR and ActR, transcriptional activator members of the Crp-Fnr regulator superfamily, control the expression of the genes encoding the Nir and Nor proteins (Baek et al., 2008). Evidence suggests that NnrR is activated by Fnr and nitric oxide (NO) (Baek et al., 2008). Indeed, the presence of nitrate or nitrite leads to low-level production of NO, which activates NnrR, in turn increasing the transcription of *nirK*. NnrR also activates *nor* expression, mitigating NO accumulation and toxicity. Small regulatory RNAs (sRNAs) are also appropriate for this finely-tuned regulation because these RNAs play critical roles in a variety of cellular processes (Harfouche et al. 2015). RNA sequencing analysis performed by

Dequivre et al. (2015) revealed the presence of a small transcript in the denitrification cluster in *A. fabrum* C58. RNA829 is located in the intergenic region of the *norCBQD* operon (Dequivre et al., 2015). However, no studies have demonstrated a biological role for sRNA in the regulation of denitrification. This work was undertaken to identify the gene that catalyses nitrate reduction and to determine the role of this gene in the fitness and rhizosphere colonization behaviour of *A. fabrum* C58. In addition, the involvement of a small transcript, RNA829, in denitrification control was studied. This work was performed by constructing a mutant that was defective in the first step of denitrification and by performing competition assays between the wild-type and mutant strains *in vitro* and *in planta*. We also measured N₂O emission in C58 strains with altered RNA829 levels and characterized the RNA829 sRNA.

2 Results

2.1 The ability to denitrify is linked to cluster 2

Two putative nitrate reductases encoding by *nasA* and *napA* are present in *Agrobacterium fabrum* C58 genome. These two genes are located in two different genomic environments on the linear chromosome. The cluster that includes *nasA* and is named “cluster 1” stretches from atu3900 (996252–998900 bp), corresponding to *nasA*, to atu3907 (1006731–1007318 bp), corresponding to *nasT* (Fig. 1b). This cluster also includes the *nrtABC* genes that encode nitrate transporters. The cluster that includes *napA* and is named “cluster 2” stretches from atu4380 (1517414 bp–1518115 bp), corresponding to *nnrR*, to atu4410 (1545294–1545995 bp), corresponding to *napC*. Cluster 2 is composed of two parts: part 1 ranges from atu4380 to atu4392 (1529019–1529696 bp), corresponding to *nnrU*, and part 2 ranges from atu4405 (1541384–1541569 bp), corresponding to *napE*, to atu4410 (Fig. 1b). This cluster harbours genes encoding known denitrifying reductases, such as the *nirK*-encoded nitrite reductase and *norC*-encoded nitrous oxide reductase. Interestingly, cluster 1 is well conserved among all 11 representative strains of 10 genomic species of the *Agrobacterium tumefaciens* complex, whereas cluster 2 is present in *Agrobacterium* sp. G2 (= *A. pusense*), *Agrobacterium* sp. G3, *Agrobacterium* sp. G4 (= *A. radiobacter*), *Agrobacterium* sp. G7 (= *A. deltaense*) and *Agrobacterium* sp. G8 (= *A. fabrum*) and absent in *Agrobacterium* sp. G1, *Agrobacterium* sp. G5, *Agrobacterium* sp. G9, *Agrobacterium* sp. G13 and *Agrobacterium* sp. G15 (= *A. viscosum*) (Table 1). Moreover, the synteny of these clusters, when they are present, is conserved (Fig. S1).

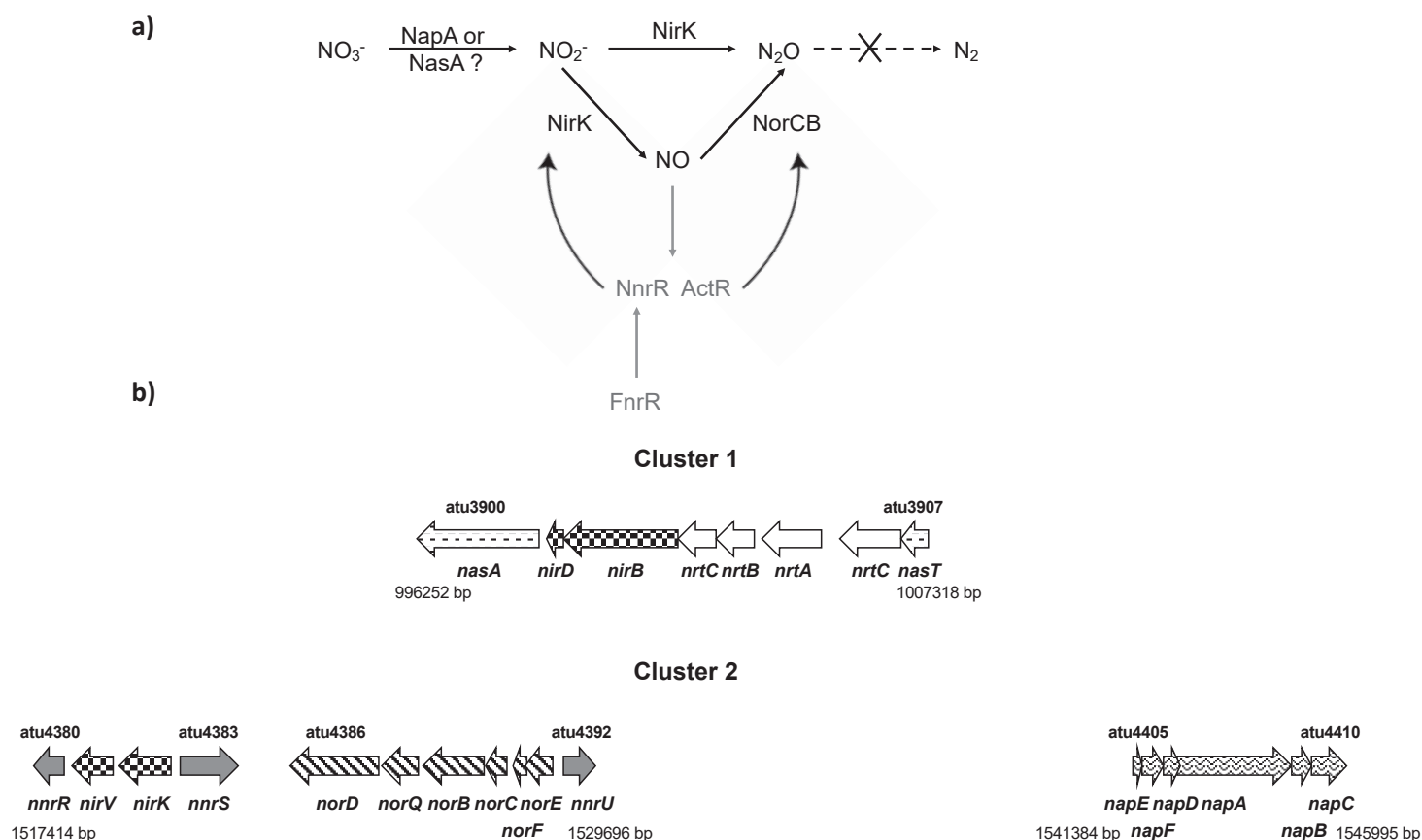


Figure 1. Denitrification pathway and its genetic organization in *Agrobacterium fabrum* C58. (a) Denitrification pathway and its regulation in *A. fabrum* C58. The reduction of soluble nitrate to nitrite is catalyzed by a nitrate reductase (NapA or NasA?). Reduction of soluble nitrite to nitric oxide gas is catalyzed by a copper (Cu-Nir) nitrite reductase. Lastly, the reduction of NO to N₂O is catalyzed by (Nor) of nitric oxide reductase. NnrR, FnrR and ActR, transcriptional regulators members of the Crp-Fnr superfamily regulators are involved in denitrification control. NnrR and ActR control the expression of the genes encoding Nir and Nor proteins (Baek & Shapleigh, 2005). Whereas, FnrR activate NnrR expression (Baek *et al.*, 2008). (b) Nitrate reductases in *A. fabrum* C58 are localized in two clusters. The cluster 1 from atu3900 (996252 – 998900 bp) to atu3907 (1006731 – 1007318 bp) includes *nasA* and *nrtABC* genes. The cluster 2 from atu4380 (1517414 bp – 1518115 bp) to atu4410 (1545294 bp – 1545995 bp) includes *nnrR*, *nirK*, *norCBQD* operon and *napEFDABC* operon. Genes involved in denitrification in *A. fabrum* C58 are localized in cluster 2. The arrows represent the gene orientation. The length of the arrows is related to the length of the genes. The genes are grouped by function and that is figured by specific filling pattern.

To determine which of the two clusters is the most important for denitrification, we studied the growth and N₂O emission of the 11 strains under denitrification conditions (Table 1). The strains belonging to the G2, G3, G4, G7 and G8 genomic species harbouring the two clusters had the ability to denitrify and could grow under denitrification conditions, whereas no denitrification and growth were detected for the strains belonging to G1, G5, G9, G13 and G15 genomic species harbouring only cluster 1 (Table 1). These results suggest that the ability to denitrify is linked to cluster 2, which includes the NapA, NirK and NorC reductases.

Table 1. Growth under denitrification conditions in *Agrobacterium* strains linked to the presence of the cluster 2. Growth and N₂O emission of one or two strains of each genomic specie belonging to the *Agrobacterium tumefaciens* complex were tested under denitrification. These strains harbor at least the cluster 1 with *nasA* gene and for some they also harbor the cluster 2 with *napA* gene. In all instances, the growth ability of *Agrobacterium* under denitrification conditions is linked to the presence of the cluster 2.

| Genomic species | Strains | Presence of cluster 1 harboring <i>nasA</i> | Presence of cluster 2 harboring <i>napA</i> | Growth and N ₂ O emission under denitrification conditions |
|--|-----------|---|---|---|
| <i>Agrobacterium</i> sp. G1 | TT111 | Yes | No | No |
| <i>Agrobacterium pusense</i> (A. sp. G2) | CFBP5494 | Yes | Yes | Yes |
| <i>Agrobacterium</i> sp. G3 | CFBP6623 | Yes | Yes | Yes |
| <i>Agrobacterium radiobacter</i> (A. sp. G4) | B6 | Yes | Yes | Yes |
| <i>Agrobacterium</i> sp. G5 | CFPB6626 | Yes | No | No |
| <i>Agrobacterium deltaense</i> (A. sp. G7) | Zutra 3-1 | Yes | Yes | Yes |
| <i>Agrobacterium fabrum</i> (A. sp. G8) | C58 | Yes | Yes | Yes |
| <i>Agrobacterium fabrum</i> (A. sp. G8) | J-07 | Yes | Yes | Yes |
| <i>Agrobacterium</i> sp. G9 | H0363 | Yes | No | No |
| <i>Agrobacterium</i> sp. G13 | CFBP6927 | Yes | No | No |
| <i>Agrobacterium viscosum</i> (A. sp. G15) | ATCC31113 | Yes | No | No |

2.2 NapA is responsible for the reduction of nitrate to nitrite

Mutant strains defective for the *napA* or *nasA* gene were constructed in the model strain *Agrobacterium fabrum* C58 and tested for their capacity to denitrify. The C58Δ*nasA* (C5802) strain was able to denitrify at the same level as C58 WT and reached 40000 ppm of N₂O emission (Fig. 2a). The *napA* deletion strain was not able to denitrify on nitrate. Moreover, C58Δ*napA* (C5801) was unable to grow on nitrate under denitrification conditions, whereas the optical density of C58 WT and C58Δ*nasA* increased 10-fold (Fig. 2a). Finally, C58Δ*napA* and C58 WT were able to denitrify and to grow on nitrite as terminal electron acceptor under anoxic conditions (Fig. 2b). The N₂O emission and the optical density were not significantly different between both strains (Fig. 2b). We propose that NapA is a nitrate reductase that is responsible for the reduction of nitrate to nitrite.

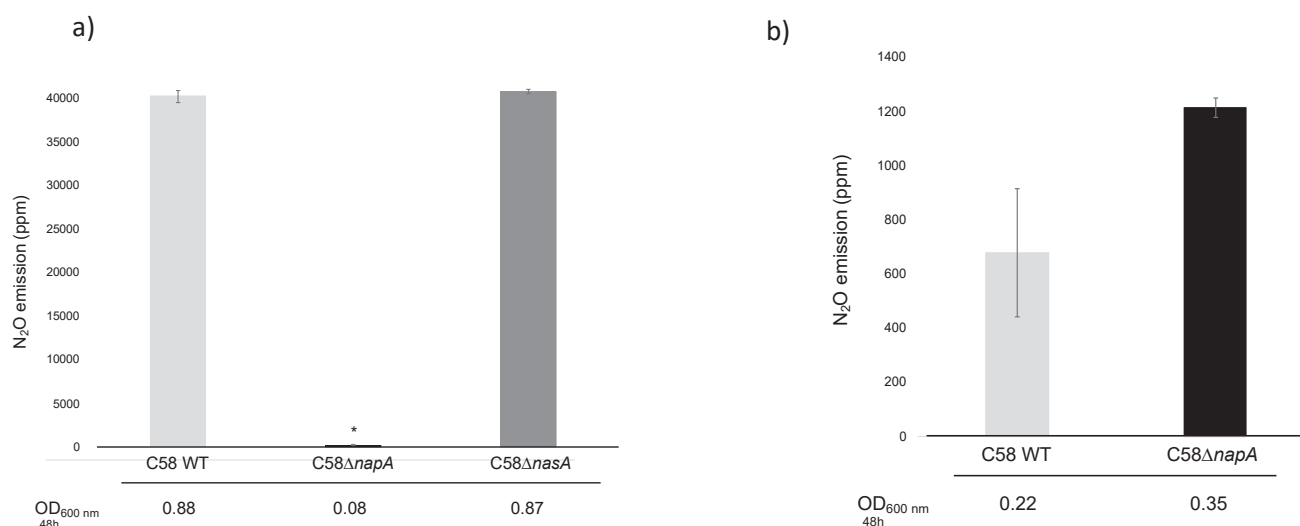
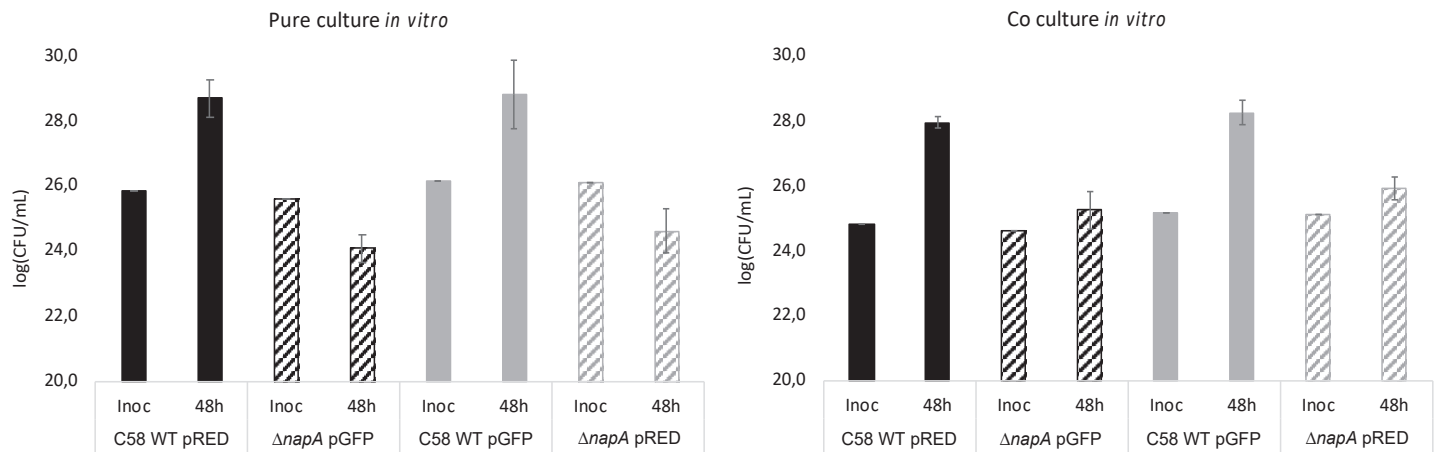


Figure 2. N₂O emission and bacterial growth after 48 hours of incubation of C58 WT, C58Δ*napA* and C58Δ*nasA* strains growing under denitrification conditions. a) On 20mM of nitrate. **b)** On 2mM of nitrite. For both, N₂O emissions were measured in ppm on strains incubated for 48 hours under denitrification conditions and in presence of nitrogen oxide. Optical density (OD_{600 nm}) indicated growth of strains. WT and strains defectives for *napA* (C58Δ*napA*) and *nasA* (C58Δ*nasA*) genes, respectively were analyzed. Error bars correspond to standard deviation. Means that differed from the WT strains is indicated by a star (Wilcoxon's test, $\alpha < 0.05$). Experiments were performed in triplicates.

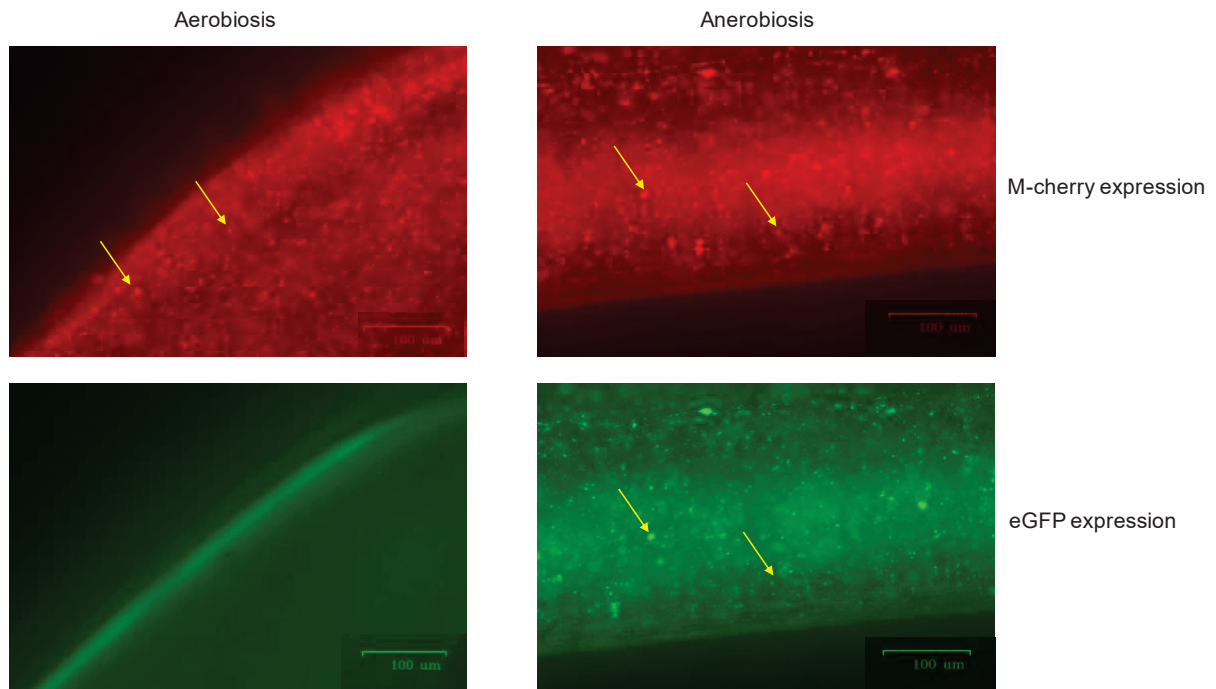
2.3 Is NapA involved in the fitness and rhizosphere colonization behaviour of *A. fabrum* C58?

To test the involvement of NapA in the fitness and rhizosphere colonization behaviour of *A. fabrum* C58 under denitrification conditions, we evaluated the growth of C58 WT and C58Δ*napA* in *in vitro* and *in planta* competition assays with strains harbouring plasmid with constitutively express fluorescent protein (GFP or Red). As expected, C58Δ*napA* was unable to grow in pure culture under denitrification conditions *in vitro*, whereas growth at 4×10^8 CFU/mL was observed for C58 WT regardless of the reporter plasmid used (Fig. 3a). The growth of C58Δ*napA* was 2-fold higher in the presence of C58 WT than in pure culture, whereas the growth of C58 WT was 2-fold lower in the presence of C58Δ*napA* than in pure culture (Fig. 3a). Nitrite level in the media was measured, however, no trace was detected. Anoxic conditions were verified *in planta* by analysing the expression of the *nirK* gene using the pOT1eM-P*nirK*::GFP reporter plasmid after 24 h, 48 h, 72 h and 9 days of bacterial inoculation. The presence of bacteria was shown by constitutive red fluorescence, and *nirK* was only expressed under anoxic conditions, as shown by inducible green fluorescence. *nirK* expression was induced as early as 24 h after bacterial inoculation (Fig. 3b). C58Δ*napA* and C58 WT grew and colonized maize roots at approximately 3×10^8 CFU/mL. This colonization was identical regardless of the growth condition: pure culture or co-inoculation under anoxic conditions (Fig. 3c).

a)



b)



c)

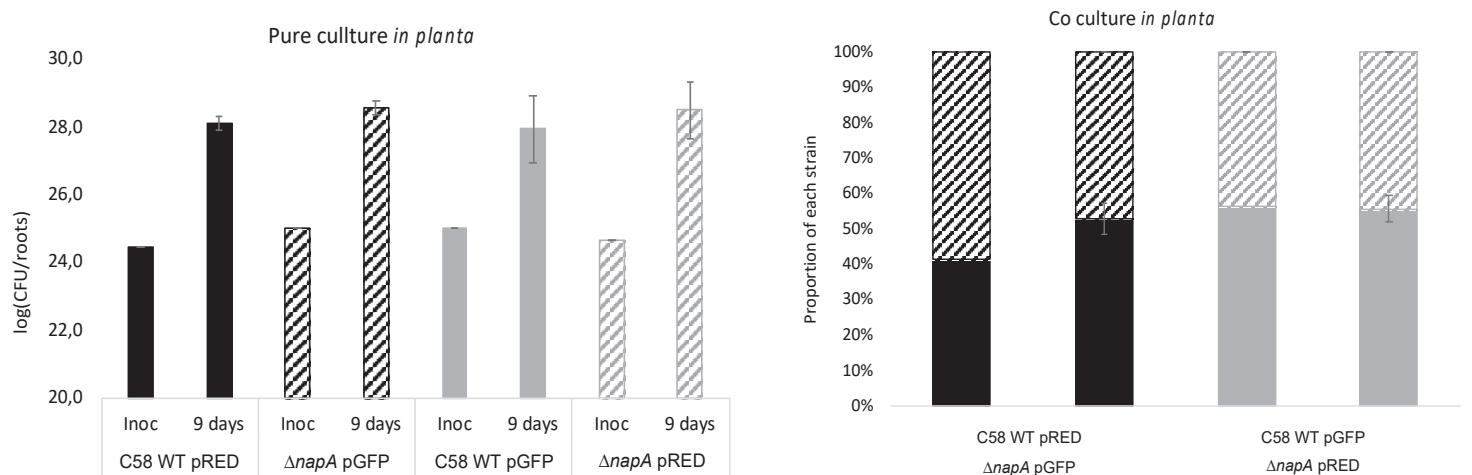


Figure 3. Competition assays between C58 WT and C58 Δ napA under anoxic conditions *in vitro* and *in planta*.
a) Growth of C58 WT and C58 Δ napA during *in vitro* assays. The graphic shows the growth of C58 WT or C58 Δ napA in pure culture and the relative abundance of C58 WT and C58 Δ napA in co-culture culture. The cell concentrations were determined from the initial inoculum and 48 h after of inoculation under anoxic conditions. Error bar correspond to standard deviation (n=3). **b)** Validation of anoxic conditions during *in planta* assays by monitoring *nirK* expression trough contact with maize roots (supplemented with nitrate) using pOT1eM-*PnirK*::GFP reporter plasmid using ZOE microscopy under oxic and anoxic conditions. Red fluorescence from m-cherry constitutive expression indicates bacterial presence (arrows as examples) whereas green fluorescence indicates active bacteria expressing *nirK* (arrows as examples). Plant auto fluorescence is represented by red and green vegetal cells. eGFP expression was intense under anoxic conditions whereas it was switch off under oxic conditions. **c)** Growth of C58 WT and C58 Δ napA during *in planta* assays. The graphic shows the growth of C58 WT or C58 Δ napA in pure culture and the relative abundance of C58 WT and C58 Δ napA in co-culture culture recovered from the maize roots. The cell concentrations were determined from the initial inoculum and 9 days of inoculation under anoxic conditions. Error bar correspond to standard deviation (n=4).

2.4 A small transcript located in cluster 2 influences the denitrification activity of *A. fabrum* C58

A small transcript, named RNA829, was previously identified on the antisense strand between the *norD* and *norQ* genes (Dequivre et al., 2015) where NorD and NorQ are chaperone proteins essential for NorCB activation (Philippot, 2002). We renamed this transcript “NopR sRNA” and tested the involvement of this sRNA in the regulation of denitrification in *A. fabrum* C58. We measured the expression level of this transcript under oxic and anoxic conditions using qRT-PCR. NopR sRNA was expressed only under anoxic conditions (data not shown). We constructed strains with altered RNA829 levels and tested these strains for N₂O emission under denitrification conditions. Every strain was able to grow under anoxic conditions, and we measured the denitrifying enzyme activities (DEAs) (Fig. 4). The DEAs of C5808 (containing a plasmid used for overexpression) and C58 NopR⁺ were 18 and 22 μ g N-N₂O/h/mL, respectively, and no significant difference was observed. The DEA of C58 NopR⁻ was also determined. In contrast, the DEA of C5810 (containing a plasmid used for RNA silencing) was significantly higher than the DEA of C58 NopR⁻, with values of 18 and 5 μ g N-N₂O/h/mL, respectively. Because the denitrification activity of C5808 was not different from that of C5810, the comparison of C58 NopR⁺ with C58 NopR⁻ was possible, and the results demonstrated a reduction of denitrification activity when NopR was silenced (Fig. 4).

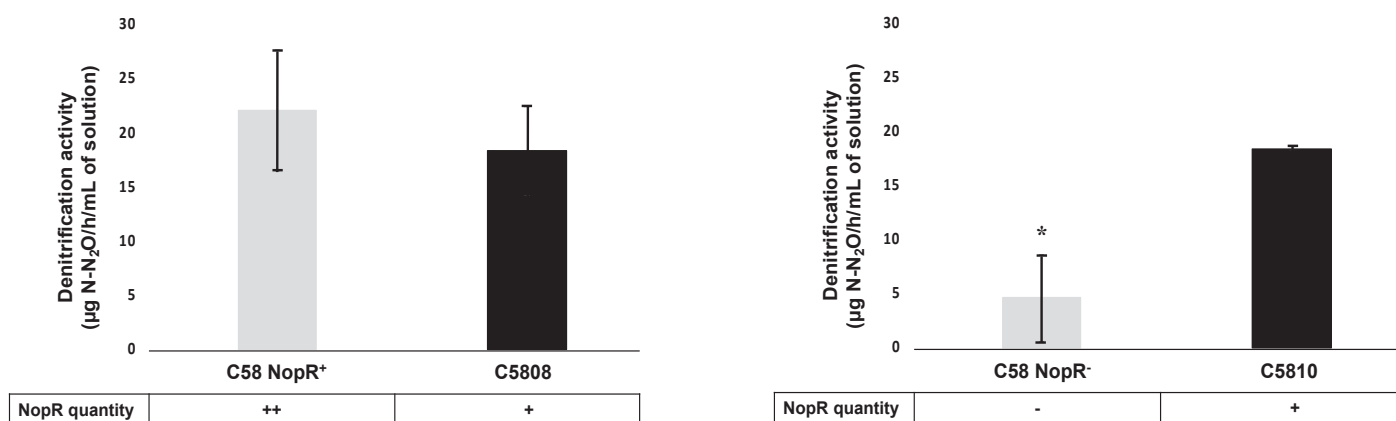


Figure 4. Denitrification activity of C5808, C58 NopR⁺, C5810 and C58 NopR⁻ strains. The graphic shows the denitrification activity (DEA, $\mu\text{g N-N}_2\text{O/h/mL}$ of solution) of C58 pBBR1-MCS2 (C5808), C58 NopR⁺, C58 pBBR1-MCS5 (C5810) and C58 NopR⁻. Quantity of NopR is indicated by (++) for C58 NopR⁺, (+) for WT strains and (-) C58 NopR⁻. Error bars correspond to standard deviation (n=6). Significant difference is indicated by a star (Wilcoxon's test, $\alpha < 0.05$).

2.5 NopR is a non-coding regulatory RNA

Using RACE PCR, we determined the 3' (1524962 bp) and 5' (1525071 bp) ends and the +1 of *nopR* transcription initiation (Fig. 5a). NopR sRNA is 80 nt long and overlap the 3'-UTR of *norQ* mRNA by about 30 pb. The -10 box (TATCCT) and -35 box (GTTCCA) were identified using a consensus sequence for *Agrobacterium spp* (Wilms et al., 2012). The RNAfold-predicted secondary structure of NopR comprises several stem loops with long stems and single-stranded loops containing several A- and G-rich regions (Fig. 5b). Considering the location of *nopR* as being cis to *norQD*, the *nopR* gene is conserved in all *Agrobacterium* strains harbouring cluster 2 of denitrification.

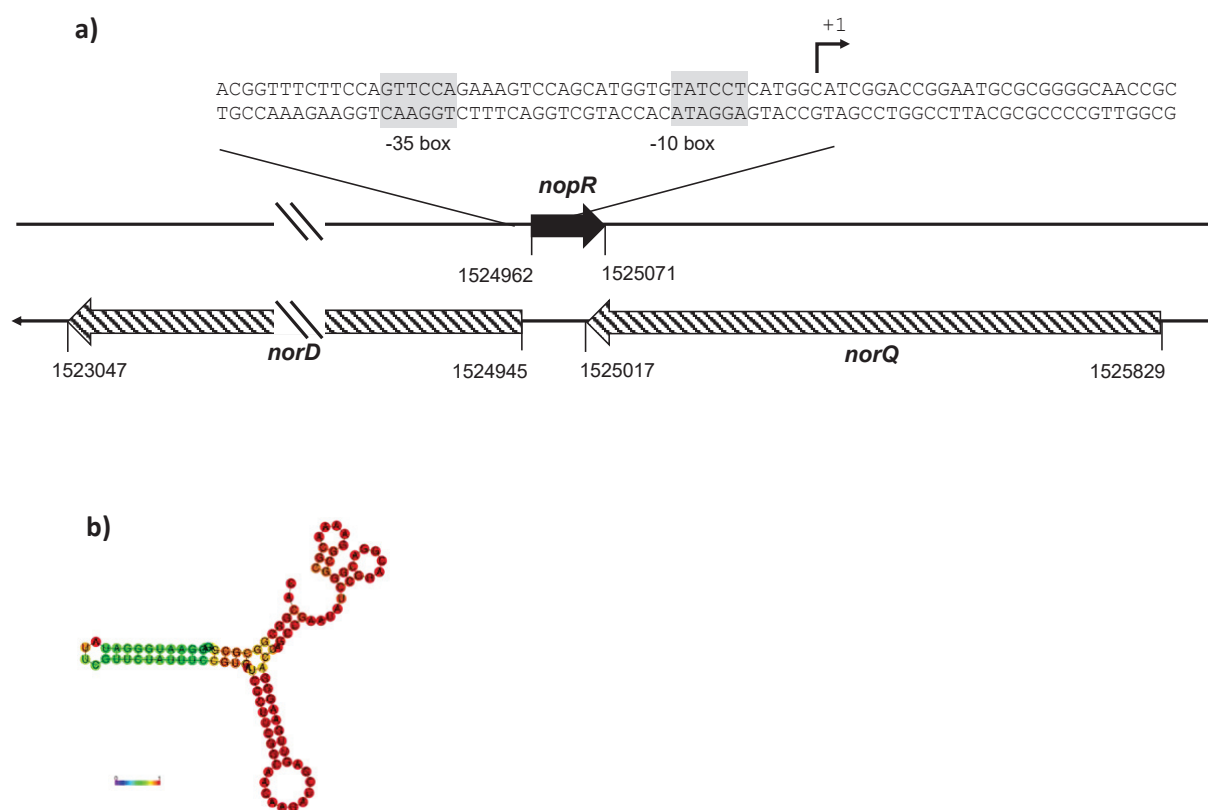


Figure 5. NopR characterization by RACE PCR and secondary structure. a) The 3' and 5' end of *nopR* sequence was identified by RACE PCR. The -10 box (TATCCT) and -35 box (GTTCCA) were identified using consensus sequence of these box known for *Agrobacterium tumefaciens* (Wilms et al. 2012). The *nopR* sequence begins at 1524962 bp and finishes at 1525071 bp. b) The minimum free energy (MFE) structure is obtained from RNAfold algorithm. The free energy of the thermodynamic ensemble is -39.32 kcal / mol. The frequency of the MFE structure in the ensemble is 26.44%. The colors represent base-pair probabilities with high value in red and low value in blue.

By measuring the amount of *norQ* mRNA using Northern Blot analysis in C58 NopR⁻ and C58 NopR⁺ strains, we evidenced the presence of *norQ* mRNA transcript (800 bp) in C58 NopR⁺ (Fig. 6a). No *norQ* mRNA transcript was evidenced in C58 NopR⁻ (Fig. 6a).

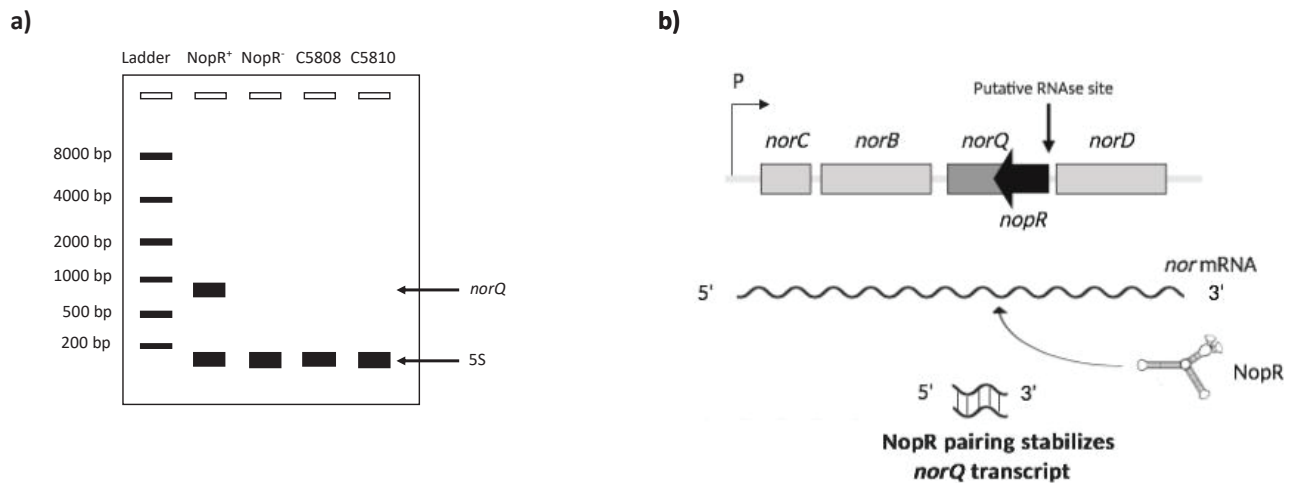


Figure 6. Model of NopR control of denitrification. a) Detection of *norQ* mRNA transcript by Northern Blot analysis using strand-specific probes for *norQ* mRNA. *norQ* mRNA transcript is only detected in C58 NopR⁺. The size of *norQ* mRNA is about 800 bp. The Northern blot was performed in two replicates for each condition. 5S rRNA served as loading control. b) Model proposal for NopR mechanism. NopR sRNA encoded on the opposite strand in the *norQ-norD* IGR may interact with *norQ* to stabilize this transcript.

3 Discussion

3.1 Denitrification is species specific in *Agrobacterium*

In biovar 1 *Agrobacterium* (also called *A. tumefaciens* species complex), two nitrate reductases may be present in two different clusters located on the linear chromosome (except for the J-07 strain, in which these genes are located on the At plasmid). The large cluster (cluster 2), including the *nir* and *nap* operons encoding nitrite, nitric oxide and periplasmic dissimilatory nitrate reductases, is well known (Baek and Shapleigh, 2005; Baek et al., 2008; Bergaust et al., 2008) and is conserved among species when present. This cluster was probably lost by the other species, as suggested by Lassalle et al. (2017). The other cluster (cluster 1), including the *nasA* and *nrt* operons encoding assimilatory nitrate reductase (Kuypers et al., 2018) and the ABC family of transporters required for nitrate transport (Luque-Almagro et al., 2017; Moreno-Vivián et al., 1999), is conserved in all species. As demonstrated, only species harbouring cluster 2 were able to denitrify, indicating that the chromosomal region from the *nnrR* to *napC* genes is the specific cluster for denitrification. Denitrification is a species-specific pathway in the *Agrobacterium tumefaciens* complex. The gene in this cluster that encodes nitrate reductase in *Agrobacterium* was not characterized, so the *napA* gene was selected as the best candidate as already suggested by Kamschreur et al. (2012) and Kuypers et al. (2018). We effectively demonstrated that the *napA* null mutant was unable to grow and denitrify

under anoxic conditions. NapA is the nitrate reductase in *A. fabrum* C58 and probably in the whole *Agrobacterium* genus.

3.2 NapA: a nitrate reductase involved *A. fabrum* C58 fitness

In soil and rhizospheric environments, bacteria need the ability to explore host resources and compete with the resident microbiota (Gonzalez-Mula et al., 2019). The denitrification pathway is one of the traits known to provide competitive advantage for rhizosphere colonization (Philippot et al., 1995; Lecomte et al., 2018). In our study, using *in vitro* assays under anoxic conditions, the mutant C58 Δ napA was unable to growth on nitrate demonstrating that NapA is involved in C58 fitness. During competition assays, we observed poor growth of C58 Δ napA simultaneously with decreased growth of C58 WT, which can be explained by the NO₂⁻ flux. Indeed, C58 WT is responsible for the reduction of NO₃⁻ to NO₂⁻, and NO₂⁻ can diffuse from cells to media. However, we couldn't detect NO₂⁻ in the media. As NO₂⁻ is toxic for cells, it is probably rapidly diverted by C58 Δ napA to aid its own growth instead of the growth of C58 WT, as demonstrated in the soil environment by Robertson and Groffman (2007). In the same ecological niches, species of *Agrobacterium* can coexist (Lassalle et al., 2011). Interestingly, species that encode the denitrification function can coexist with those lacking this function. This finding suggests a Black Queen Hypothesis (BQH) context in which some *Agrobacterium* species called "helpers", as these species provide the denitrification function, build obligate associations with species that avoid the denitrification function, called "beneficiaries" (Morris et al., 2012; Zomorodi and Segrè, 2016; Mas et al., 2016). In addition, because helpers provide nitrite for the growth of beneficiaries under certain conditions, these species also produce NO, which may play a role in efficient plant colonization (Ghiglione et al., 2000), probably *via* protection of roots against phytopathogenic competitors (Compant et al., 2010).

3.3 NapA is not involved in *A. fabrum* C58 rhizosphere competence

For *in planta* competition assays, we chose *Zea mays* plants that exude different carbon sources, such as rhamnose, maltose, succinate and citric acid (Krafczyk et al., 1984), and are efficiently colonized by *Agrobacterium* species (Chèneby et al., 2004). Surprisingly, no differences in growth were observed between C58 WT and C58 Δ napA and in the competition assays during *in planta* experiments under anoxic conditions. This finding suggests that (i) NapA is not involved in C58 fitness during *in planta* growth, (ii) C58 Δ napA could alternatively use exudates as terminal electron acceptors, allowing the growth of this strain and favouring root colonization under anoxic conditions. Indeed, humic substances, the major component of soil's organic matter and plants exuded primary metabolites, such as fumaric acid could be used by microorganisms for anaerobic growth (Guyonnet et al., 2017; Lecomte et al., 2018).

3.4 NopR is involved in denitrification control

To adapt to environmental changes, such as rapid transition from oxic to anoxic conditions, coordination of the regulation and activity of the different reductases involved in denitrification is necessary and may be mediated by non-coding RNA. The role of non-coding RNAs in the repression of metabolic enzymes that have aerobic functions under low-oxygen conditions was already demonstrated in *Enterobacteria* (Boysen et al., 2010; Durand and Storz, 2010). However, nothing is known regarding the activation of the denitrification process by non-coding RNA in *Agrobacterium*. In this study, we discovered a novel sRNA named NopR (denitrification regulation sRNA) that is involved in the activation of denitrification in the C58 strain. NopR is a cis-encoded antisense sRNA that is located on the opposite strand of the *norQD* genes, expressed under anoxic conditions and is conserved among genomic species harbouring cluster 2. We evidenced that *norQ* mRNA level is increased concomitantly with N₂O emissions in C58 NopR⁺ strain. Therefore, we suggest that during anoxic condition, NopR forms base pairs with the 3' end of the *norQ* mRNA and confers increased in *norQ* mRNA stability allowing for *norQ* mRNA accumulation and the increased in N₂O emission via NorC activation (Fig. 6b). Indeed, putative RNase E site (Krinke & Wulff, 1990) has been found between *norQ* and *norD*. Base pairing between NopR and *norQ* mRNA could impede the binding or activity of an RNase that functions at the 3' end of the *norQ* mRNA as already described for GadY in *E. coli* (Opdyke et al., 2004). Interestingly, NopR is the second sRNA suggested to act by stabilising mRNA. Indeed, *cis*-encoded antisens RNAs have been reported to repress mRNA, with one exception: GadY (Opdyke et al., 2004).

Future studies will identify plant components that could be used as terminal electron acceptors by *A. fabrum* C58 or by *Agrobacterium* strains lacking the denitrification function, allowing growth and favouring root colonization in anoxic conditions.

4 Experimental procedures

4.1 Bacterial strains and growth conditions

Escherichia coli and *Agrobacterium* strains used in this study are summarized in Table S1. *Agrobacterium fabrum* C58 wild-type and its derivatives were grown aerobically at 28°C with shaking (180 rpm) on YPG rich media (per liter: 5 g of yeast extract, 5 g of peptone, 10 g of glucose, pH 7,2). Media were supplemented, when needed, with appropriate antibiotics (100 µg/mL of ampicillin, 30 µg/mL of gentamycin, 25 µg/mL of kanamycin and 25 µg/mL of neomycin, 10 µg/mL of tetracycline). In denitrification conditions, *A. fabrum* C58 and its derivatives were grown on YPG supplemented with

20 mM of KNO_3^- at 28°C without shaking in 160 mL flasks closed hermetically with corks. 140 mL of helium were injected into flasks to attempt anoxic conditions.

4.2 Construction of mutant and fluorescent strain

4.2.1 C58 Δ *napA* and C58 Δ *nasA*

The linear chromosome DNA fragments corresponding to *nasA* (atu3900) and *napA* (atu4408) were deleted by double-recombination from C58 WT as described by Lassalle and al. (2011), resulting in C58 Δ *nasA* and C58 Δ *napA* strains (Table S1). Briefly, mutagenic PCR fragments were created by joining two fragments corresponding to the two regions flanking the sequence to be deleted (average 1kb each) using In-Fusion cloning kit (Clontech lab) (Table S2). This fragment was cloned into the pre-open pJQ200SK, a plasmid carrying the gentamycin resistance cassette and the *sacB* gene conferring sucrose sensitivity (Quandt & Hynes, 1993) (Table S1). The resulting plasmid was introduced in C58 WT by electroporation (Lassalle et al., 2011). Single recombinants were selected on YPG media supplemented with gentamycin. Double recombinants were selected on YPG media supplemented with 5% sucrose. Deletion mutants were verified by PCR with appropriate primers and Sanger sequenced.

4.2.2 Overexpression and reduced expression of NopR

The linear chromosomal DNA fragment corresponding to *nopR* (from 1524962 bp to 1525071 bp) was amplified by PCR using two different couples of primers (Table S2). The fragments were digested using the HindIII and BamHI enzymes and were then ligated into the pBBR1MCS-2 and pBBR1MCS-5 plasmids, respectively, pre-linearized with the same enzymes, resulting in the *pnopR*⁺ and *pnopR*⁻ plasmids. The total sequence of *nopR* was constitutively expressed under plasmid promoter control from the plasmid *pnopR*⁺, leading to overexpression of NopR. In contrast, a fragment of the *nopR* sequence was constitutively expressed under plasmid promoter control from the plasmid *pnopR*⁻, leading to hybridization of genomic NopR with plasmid NopR and decreased NopR levels. Electroporation of the *pnopR*⁺ and *pnopR*⁻ stable plasmids into *A. fabrum* C58 yielded C58 NopR⁺ and C58 NopR⁻, respectively. The pBBR1MCS-2 and pBBR1MCS-5 empty plasmids were also introduced by electroporation into the *A. fabrum* C58 strain as a control.

4.2.3 Construction of *nirK* translational fusion

To construct translation eGFP fusion to *nirK*, fragment containing the *nirK* promoter region and the translation initiation sites was amplified by PCR using the appropriate primers (Table S2) and chromosomal DNA of strain C58 as template. The fragment was digested with *Apal-SpeI* and cloned into the corresponding sites of pOT1eM plasmid in-frame of *egfp* generating pOT1eM-*PnirK*::GFP. pOT1eM plasmid contains also *m-cherry* gene. The constitutively expressed *m-cherry* gene (red color) indicates the bacteria presence whereas the inducible expressed *egfp* gene (green color) reports expression of *nirK*.

4.3 Denitrification activity

A. fabrum strains were grown under denitrification conditions (anaerobically on YPG media supplemented with 20 mM KNO₃). Flasks were inoculated at 4.2.10⁷ bacterial cells/mL from a 48 hours anoxic culture on YPG supplemented with 20 mM of NO₃⁻ or with 2 mM of NO₂⁻. Denitrification activities were performed by measuring N₂O accumulation over the time (every 4 hours) using gas chromatograph coupled to a micro-catharometer detector (μGC-R3000, SRA instruments, Marcy l'Etoile, France) as previously describe by Guyonnet et al. (2017). Experiments were performed during 48 h in order to attempt stationary phase of N₂O emission. Each experiment was performed in triplicates.

4.4 RNA characterization

4.4.1 RNA isolation

Strains were grown under denitrification conditions for 24 hours (corresponding to the beginning of the stationary phase of denitrification activity). Cells were harvested and total RNAs were extracted by phenol chloroform method as previously described by Hommais et al. (2008). DNA traces was eliminated with two DNase I treatments (DNA-free kit, Ambion). Isolated RNA was quantified using Nanodrop spectrophotometer (NP80, Implen), visualized on an agarose gel to check quality, and stored at -80°C. Each experiment was performed in triplicates.

4.4.2 Quantitative real-time reverse-transcription PCR (qRT-PCR)

2.5 μg of total DNA-free RNAs were reverse transcribed using random hexamer primer according to manufacturer's recommendations (ABM). qRT-PCR were performed using the SYBR Green PCR Master mix kit (Thermo Scientific) as previously described by Dequivre et al. (2015). LC480 Lightcycler from Roche was used for thermal cycling reactions according to the following protocol: an initial step at 95°C for 10 min, followed by 45 cycles at 95°C for 10 s, 58°C for 30 s, and 72°C for 20 s in order to amplify *nopR* (Table S2). Normalization of genes expression was performed using *rpoD* and *atpD* housekeeping genes. Melting curves were checked in order to verify PCR primer specificity.

4.4.3 Northern Blot

10 μg of total RNAs extracted from C58 NopR⁺, C58 NopR⁻, C5808 and C5810 and 2 μg of ^{DynaMarker} RNA High ladder (BioDynamics Laboratory Inc) were used for electrophoresis performed on 1% agarose gel containing 25 mM guanidium thiocyanate and ethidium bromide. After migration, RNAs were transferred by capillary action on nitrocellulose membrane. Hybridization was achieved with specific digoxigenin (DIG)-labelled probes complementary to the intergenic region of *norQ* and *norD* or to the 5S rRNA. Anti-digoxigenin-AP antibody (ROCHE) was used conjugated with CSPD substrate (ROCHE),

and chemiluminescent detection was carried out with the FUSION-FX7 Spectra (Vilber). The Northern blot analysis was performed in two replicates for each condition.

4.4.4 Determination of 5' and 3' ends by RACE PCR

RACE-PCR was performed as previously described by Dequivre et al. (2015). Briefly, 12 µg of total DNA-free RNA were treated successively with different enzymes as follow: the XRN-I exoribonuclease (Biolabs) were used to eliminated cleaved monophosphate RNAs, then tobacco acid pyrophosphatase (Epicentre) were used to treat the resulting triphosphate RNAs and finally T4 RNA ligase (Biolabs) were used to circularize RNAs. EasyScript Reverse Transcriptase kit were used to reverse transcribe 2.5 µg of total circular DNA-free RNA using primers specific to the gene *nopR* (Table S2). The 5' and 3' end junction was specifically amplified by PCR. The products were cloned into pGEMT-Easy (Promega) and were Sanger sequenced using M13fwd primer. Five independent clones were analyzed.

4.4.5 Secondary structure

Secondary structure was predicted from *nopR* gene sequence obtained by RACE-PCR using basic options of RNAfold algorithm (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>) as previously describe by Dequivre et al. (2015).

4.5 Competition assays

4.5.1 *In vitro*

A culture of only one strain (C58 WT or C58Δ*napA*) or a co-culture of both strains (C58 WT and C58Δ*napA*) containing a plasmid with constitutively express fluorescent protein (pRED or pGFP) at 1:1 ratio was grown under denitrification conditions (Table S1). After 48h of growth, the absolute number of colonies forming units (CFUs) of WT and mutant strains were counted by plating these bacterial suspensions on YPG supplemented with tetracycline. The colonies, colored in red or green according to the harbored plasmid, were differentiated by eyes. The two combinations of reporter plasmid were performed to verify that no effect was noticeable on bacterial fitness.

4.5.2 *In planta*

The surfaces of maize seeds were disinfected by immersion in bleach and washing in ultra-pure water. For pre-germination, seeds were plated on agar for plant culture at 8 g/L (Sigma Aldrich) and incubated in the dark for 48 h at 28°C. The germinated seeds were then transferred in glass tubes, tightened approximately 5 cm from the bottom, containing 5 mL of plant nutrient solution supplemented with 5 mM nitrate. Then, these seeds were grown in a climatic chamber for 5 days at 24°C and 18°C with a 16/8-hour day/night cycle and 70% humidity. Five-day seedlings were inoculated with bacterial cell suspensions containing either only one strain (C58 or C58Δ*napA*) or a mixture of both strains (C58 and C58Δ*napA*) harbouring the pRED or pGFP reporter plasmid at a 1:1 ratio. Then, these inoculated 5-day seedlings were grown for 9 days under anoxic conditions by hermetically sealing the tubes with corks.

No carbon sources were added. Anoxic conditions were tested by inoculating C58 pOT1eM-*PnirK*::GFP *in planta* supplemented with 20 mM of nitrate and visualizing fluorescence expression by microscopy (ZOE, fluorescent cell imager). Red fluorescence from constitutively expressed m-Cherry indicates bacterial presence, whereas green fluorescence indicates active bacteria expressing *nirK* only under anoxic conditions. After 9 days, the colonizing bacteria were recovered. Roots were crushed for 5 min with metallic balls and 5 mL of water to recover bacterial cells, which were plated on YPG supplemented with tetracycline. Bacterial enumeration was performed as previously described. Two combinations of reporter plasmids were used to verify that there was no noticeable effect on bacterial fitness.

4.6 Similarity sequence analysis

Comparative genome analysis was performed on the whole genomes of *Agrobacterium* genus available on MAGE database (Vallenet et al., 2017) to study the synteny of denitrification genes and the conservation of NopR non-coding RNA in this genus.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We acknowledge funding from “La Fédération de Recherche (FR) BioEnviS, Université Lyon 1” and the French National Research Agency (ANR-18-CE32-0005, DIORE). S. Lecomte received doctoral grants from the French Ministère de l’Education nationale de l’Enseignement Supérieur et de la Recherche. Quantitative PCR were performed at the DTAMB platform (FR BioEnviS, University Lyon 1), plants were grown at the greenhouse platform (FR BioEnviS, University Lyon 1) and N₂O emission were measured at the AME platform (UMR 5557, University Lyon 1).

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Supplementary Figures and Tables

Table S1. Bacterial strains and plasmids used in this study

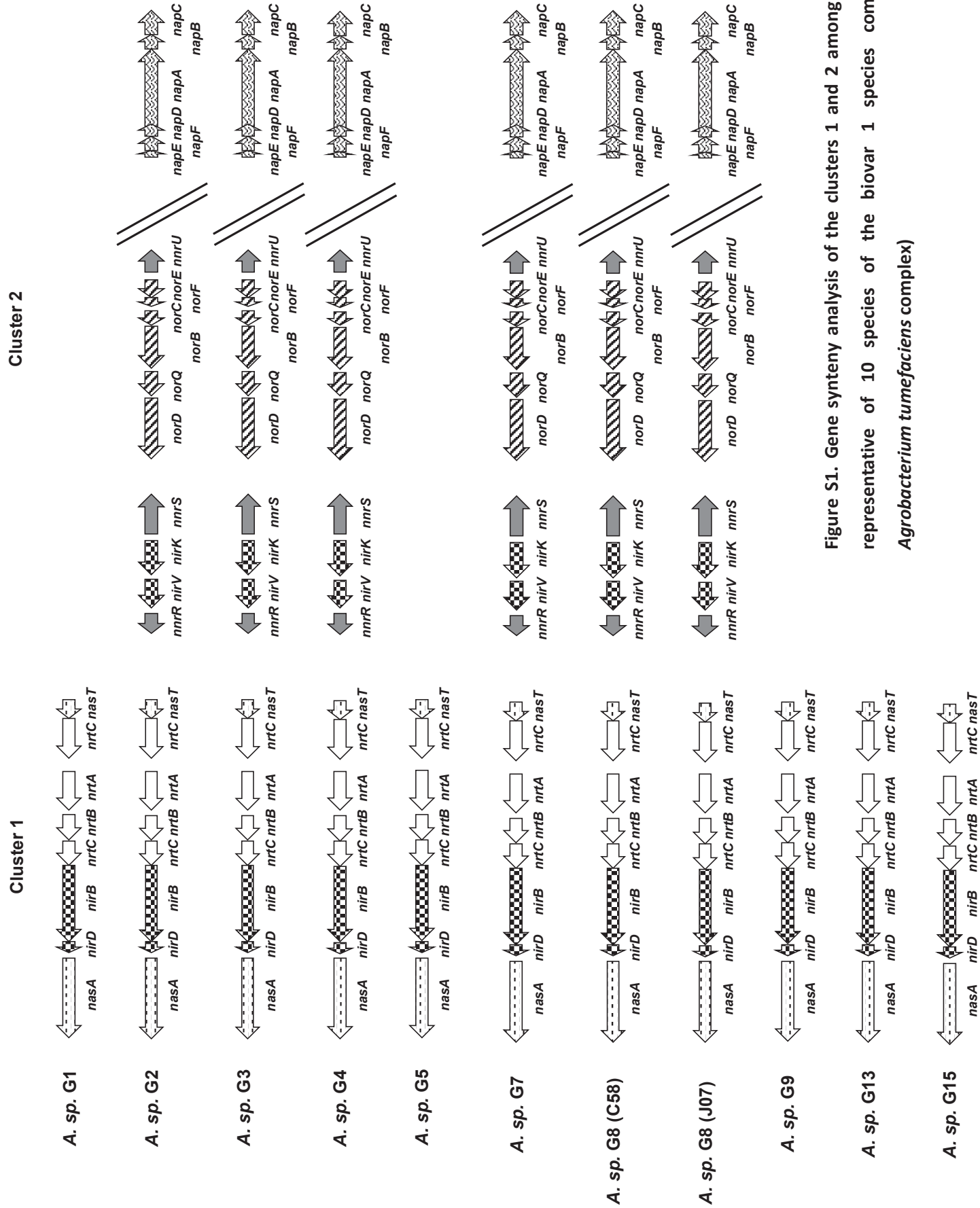
| Strains | Relevant genotype and description | Reference or source |
|---------------------------------------|---|------------------------|
| <i>Escherichia coli</i> | | |
| DH5α | <i>E. coli</i> strain used for over and reduce expression of NopR | NEB catalog |
| Stellar | <i>E. coli</i> strain used for <i>napA</i> and <i>nasA</i> mutant construction | Takara catalog |
| <i>Agrobacterium fabrum</i> | | |
| C58 WT | <i>Agrobacterium fabrum</i> C58 wild type | CFBP 1903 |
| C5801 | C58 deleted of <i>napA</i> (atu4408) gene | This study |
| C5802 | C58 deleted of <i>nasA</i> (atu3900) gene | This study |
| C5803 | C58 harboring pME6010::GFP plasmid | This study |
| C5804 | C58 harboring pME6010::RED plasmid | This study |
| C5805 | C58Δ <i>napA</i> harboring pME6010::GFP plasmid | This study |
| C5806 | C58Δ <i>napA</i> harboring pME6010::RED plasmid | This study |
| C5807 | C58 harboring pOT1eM- <i>PnirK</i> ::GFP plasmid | This study |
| C5808 | C58 harboring pBBR1-MCS2 empty plasmid | This study |
| C5809 | C58 harboring pBBR1-MCS2:: <i>nopR</i> ⁺ plasmid | This study |
| C5810 | C58 harboring pBBR1-MCS5 empty plasmid | This study |
| C5811 | C58 harboring pBBR1-MCS5:: <i>nopR</i> ⁻ plasmid | This study |
| <hr/> | | |
| Plasmid | Description | Reference or source |
| pJQ200SK | Suicide vector; P15A <i>sacB</i> (used for mutant construction); Gm ^R | Quandt and Hynes, 1993 |
| pME6010 | Stable vector (used for reporter plasmid construction); Tet ^R | Heeb et al., 2000 |
| pOT1eM | pOT1e derivative harboring <i>Ptac-m-cherry</i> inserted in <i>Clal-Sall</i> site (used for fluorescent reporter protein); Gm ^R | Meyer et al., 2018 |
| pJQ200SK:: <i>napA</i> | pJQ200SK:: <i>napA</i> ; 600 pb upstream and downstream regions of <i>napA</i> gene inserted in <i>SmaI</i> site in pJQ200SK; Gm ^R | This study |
| pJQ200SK:: <i>nasA</i> | pJQ200SK:: <i>nasA</i> ; 1 kb upstream and downstream regions of <i>nasA</i> gene inserted in <i>SmaI</i> site in pJQ200SK; Gm ^R | This study |
| pBBR1-MCS2 | pBBR1-MCS2 stable vector; Neo ^R (used for RNA overexpression construction) | Kovach et al., 1994 |
| pBBR1-MCS5 | pBBR1-MCS5 stable vector; Gm ^R (used for RNA silencing construction) | Kovach et al. 1994 |
| pBBR1-MCS2:: <i>nopR</i> ⁺ | pBBR1-MCS2 carrying <i>nopR</i> region inserted in <i>BamHI-HindIII</i> site; Neo ^R | This study |
| pBBR1-MCS5:: <i>nopR</i> ⁻ | pBBR1-MCS5 carrying <i>nopR</i> region inserted in <i>BamHI-HindIII</i> site; Gm ^R | This study |

| | | |
|----------------------------|---|------------------------------|
| pME6010::GFP | pME6010 carrying gene encoding GFP fluorochrome inserted in <i>XhoI</i> site; Tet ^R | Meyer et al., Unpublished |
| pME6010::RED | pME6010 carrying gene encoding RED fluorochrome inserted in <i>XhoI</i> site; Tet ^R | Meyer et al., Unpublished |
| pOT1eM- <i>PnirK</i> ::GFP | pOT1eM- <i>PnirK</i> ::GFP; promoting <i>nirK</i> region inserted in <i>Apal-SpeI</i> site in pOT1eM in front of <i>gfp</i> gene; Gm ^R | This study |

Table S2. Primers used in this study

| Target | Name | Sequence (5'-3') | Reference |
|--|----------------------------|---|------------|
| Deletion mutants | | | |
| <i>napA</i> flanking upstream region | <i>napA</i> UP F | ACTAGTGGATCCCCAAAATGCGTTGAAGCCTGTC | This study |
| | <i>napA</i> UP R | TTCCGCAAACAGGACCATCGCCGCCTGTCTC | |
| <i>napA</i> flanking downstream region | <i>napA</i> DW F | GTCCTGTTTGCGGAAGTCGCATGATCAGGAAACAC | |
| | <i>napA</i> DW R | GAATTCCTGCAGCCCCAAATCTTGCCCCAGACTTC | |
| <i>nasA</i> flanking upstream region | <i>nasA</i> UP F | ACTAGTGGATCCCCACCTCGACCGGGATATTTTC | This study |
| | <i>nasA</i> UP R | TTCCGCAAACAGGACAAACCGCAGCGGAATGAAC | |
| <i>nasA</i> flanking downstream region | <i>nasA</i> DW F | GTCCTGTTTGCGGAATTCAGCGGGCATCGAGTT | |
| | <i>nasA</i> DW R | GAATTCCTGCAGCCCAGGATATCGGCGTCATCTGC | |
| <hr/> | | | |
| <i>nirK</i> translational fusion | | | This study |
| <i>nirK</i> promoting region | <i>nirK</i> fusion F | ACTACTAGTTCATGTTTCTCCTGTCCCTCT | |
| | <i>nirK</i> fusion R | GGGGGGCCCTATAGTGCTCCTTGCTTGGTCT | |
| <hr/> | | | |
| NopR characterization | | | |
| <i>nopR</i> | <i>nopR</i> F | GGTTGCCCCGCGCATTC | This study |
| | <i>nopR</i> R | GTGTGGATATCAAAGCGCGCTA | |
| <hr/> | | | |
| NopR constructions | | | |
| <i>nopR</i> ⁺ insertion into pBBR1-MCS2 | <i>nopR</i> ⁺ F | CACGGTACCGTTTCGCCGATCAGCCTG | This study |
| | <i>nopR</i> ⁺ R | CACAAGCTTACCATGATCGAACCGCTGA | |
| <i>nopR</i> ⁻ insertion into pBBR1-MCS5 | <i>nopR</i> ⁻ F | CACGGTACCCGCTTTGATATCCACACCGG | This study |
| | <i>nopR</i> ⁻ R | CACAAGCTTCGGAAACAGATGGTCAGCAC | |
| <hr/> | | | |
| Northern Blot | | | |
| norD probe | <i>norD</i> F | CCTTTATCCTCAACCGCTTC | This study |
| | <i>norD</i> R | TAATACGACTCACTATAGGGTAATCCCATTCTGGGATAGGT | |

| | | | |
|---------------|---------------|--|------------|
| norQ probe | <i>norQ</i> F | ATGGACGGTCCTCTGACA | This study |
| | <i>norQ</i> R | TAATACGACTCACTATAGGGGTTCTGGTATCCGGGGTTAT | |
| norC probe | <i>norC</i> F | AAACACGTGTGGGAGAAAAA | This study |
| | <i>norC</i> R | TAATACGACTCACTATAGGGGTTCTGTTCCGGTGAGATTG | |
| <hr/> | | | |
| RTqPCR assays | | | |
| <hr/> | | | |
| <i>nopR</i> | <i>nopR</i> F | GGTTGCCCCGCGCATTG | This study |
| | <i>nopR</i> R | GTGTGGATATCAAAGCGCGCTA | |
| <i>atpD</i> | <i>atpD</i> F | AACTGATCAACAACGTCGCC | This study |
| | <i>atpD</i> R | GTTCGTTTCATCTGGCCGTAC | |
| <i>rpoD</i> | <i>rpoD</i> F | AGGAGCGTCGTTACAAGGAA | This study |
| | <i>rpoD</i> R | GCTTGACGCCATAGGATTG | |



Chapter 2

Strategy for the identification of *Agrobacterium fabrum* C58 genes crucial for denitrification *in vitro* and *in planta*

Preamble

The rhizosphere competence is defined as the genetic and phenotypic traits that ensure microbial colonization of the rhizosphere (González-Mula et al., 2018). In the rhizosphere, when the partial pressure of O₂ become low and carbon sources and nitrate are present, denitrification function is prevalent. In Pseudomonads, **denitrification is considered as a rhizosphere competence trait** (Ghirardi et al., 2012). In this instance, harboring denitrification genes could allow bacteria to preferentially colonize plant roots. Notably, ***Agrobacterium fabrum* C58 is able to colonize maize rhizosphere and reduce successively nitrate to nitrite, nitric oxide and finally nitrous oxide** using NapA (Lecomte *et al.*, submitted), NirK and NorC (Baek & Shapleigh, 2005) reductases, respectively.

This works aimed to evidence denitrification regulatory network *in vitro* and *in planta* and to highlight its role in *A. fabrum* C58 fitness and maize roots colonization. We first constructed an *A. fabrum* C58 transposon mutant library. Then, we **identify genes involved in denitrification** by performing Tn-seq experiment: (i) *in vitro* using **nitrate and nitrite as terminal electron acceptors** under anoxic condition and (ii) *in planta* using maize host to **determine whether denitrification is a rhizosphere competence trait**.

We demonstrated **a high saturation and a good quality** of *A. fabrum* C58 transposon library. We revealed that 10 and 3 % of circular and linear chromosome are dedicated to essential genes, respectively. **These genes are involved in DNA replication, protein translation, cell growth and division, and core metabolic pathways**. We also evidenced two conserved genomic regions without essential gene that were probably acquired at an early stage of *A. tumefaciens* complex history.

To identify *A. fabrum* C58 crucial genes for denitrification *in vitro* and in maize root colonization, we **set up and performed a Tn-seq experiment *in vitro* and *in planta* under denitrification condition**. Denitrification is inducible function that need absence of oxygen and presence of carbon and nitrogen sources. During our experiments, anoxic condition was applied, nitrogen source was either nitrate or nitrite and carbon source was either glucose or root exudates. The first part of Tn-seq experiment corresponding to **growth of the transposon populations on nitrate, nitrite and maize root under anoxia** was done successfully. However, the second part corresponding to **high-throughput sequencing of DNA library** is still under progress.

Results of this work are organized in a manuscript.

Strategy for the identification of *Agrobacterium fabrum* C58 genes crucial for denitrification *in vitro* and *in planta*

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Summary

- *Agrobacterium fabrum* C58 is a root associated bacterium able to perform denitrification function by reducing successively nitrate to nitrite, nitric oxide and finally nitrous oxide using NapA, NirK and NorC reductases, respectively. Nevertheless, direct evidence of the contribution of denitrification to *A. fabrum* C58 fitness and root colonization was still missing.
- We used Tn-seq analysis which combines transposon insertional mutagenesis with high sequencing of the transposon mutant populations to identifying denitrification pathway regulation *in vitro* and in association with maize host.
- The saturation of our library was high and ensured the revelation of more than 350 essential genes in *A. fabrum* C58 genome. We highlighted two conserved genomic regions that were probably acquired at an early stage of *Agrobacterium tumefaciens* complex history. We provide evidence that genes harbored by Ti plasmid required for virulence *in planta* have high metabolic costs for *A. fabrum* C58 *in vitro*.
- This work is still under progress and will confirm that denitrification is a rhizosphere competence trait in *A. fabrum* C58. It further identifies the crucial genes for denitrification *in vitro* and *in planta* by mutagenesis experiments and competitions assays.

Key words

Agrobacterium fabrum C58, anoxic root colonization, denitrification, gene identification, Tn-seq

1 Introduction

The rhizosphere, volume of soil around living roots (Hiltner, 1904), is an important microbial hotspot in which number of biotic and abiotic parameters are involved in shaping microbial-plant interactions, such as root exudates, pH and oxygen availability. Microorganisms are able to colonize the rhizosphere when they possess genetic and phenotypic traits referred as rhizosphere competence (González-Mula et al., 2018). Interestingly, denitrification, the microbial anaerobic respiration of nitrogen oxides, is considered as a rhizosphere competence trait (Ghirardi et al., 2012). The denitrification function is prevalent once the appropriate conditions are available (low or no O₂ concentration, and easily available organic compounds as energy sources and the presence of nitrate). In plant rhizospheres, the roots of growing plants cannot only release carbon compounds *via* exudation but also modify the soil oxygen partial pressure and the nitrate concentration (Lecomte et al., 2018; Achouak et al., 2019). During denitrification, a variety of intermediate compounds are produced as nitrate is reduced stepwise to N₂, including the nitrite (NO₂⁻), and the gases nitric oxide (NO) and nitrous oxide (N₂O) (Zumft, 1997). Remarkably, certain of these intermediates such as NO, can also act as signaling molecule during bacterial-root association (Vaishnav et al., 2018). Denitrifiers are able to detoxify the NO produced by plants using denitrification genes for full root colonization (Dalsing et al., 2015) and to produce it in order to compete with other microbes for root colonization (Compant et al., 2010). Collectively, these findings suggest that the ability to denitrify could be significant for the survival and growth of free-living and plant-associated rhizobacteria.

Among denitrifying rhizobacteria, *Agrobacterium fabrum* C58, a root associated bacterium, is able to reduce successively nitrate to nitrite, nitric oxide and finally nitrous oxide using NapA (Lecomte et al., chapter 1), NirK and NorC (Baek & Shapleigh, 2005) reductases, respectively. *A. fabrum* C58 is a partial denitrifier lacking NosZ reductase and participating to N₂O emission (a greenhouse gas) from the rhizosphere. While the reductases involved in *A. fabrum* C58 denitrification are well characterized, few information is available about denitrification regulation *in vitro* and *in planta* and its role in root colonization. Indeed, it is well known that the transcriptional activator members of the Crp-Fnr regulator superfamily, NnrR and ActR, control the expression of the genes encoding the Nir and Nor proteins (Baek et al., 2008). Recently, we also evidenced the regulation of Nor by a small non-coding RNA, NopR (Lecomte et al., chapter 1). *A. fabrum* C58 is known to colonize plant rhizosphere such as maize (Chèneby et al., 2004) and to denitrify in *Arabidopsis thaliana* leaves (Baek & Shapleigh, 2005). However, direct evidence of the contribution of denitrification to *A. fabrum* C58 fitness and root colonization was still missing. Identifying denitrification pathway regulation represent important issues in ecology for understanding bacterial fitness and plant root colonization.

The aim of this work was to provide a clear picture of denitrification regulatory network and highlight its role in *A. fabrum* C58 fitness and maize roots colonization. For this, we opted for Tn-seq

analysis which combines transposon insertional mutagenesis with high-throughput sequencing of the transposon mutant populations. First, the transposon mutant library was constructed. Then, libraries were tested under denitrification condition *in vitro* with nitrate or nitrite as terminal electron acceptors (TEAs) and *in planta* on maize roots with nitrate as TEA. We demonstrated that the quality of the transposon mutant library was very good. We revealed more than 350 essential genes in *A. fabrum* C58 genome carried by the circular and linear chromosomes and involved DNA replication, protein translation, cell growth and division, and core metabolic pathways. Identification of genes required for (i) denitrification respiration on nitrate and nitrite *in vitro* and (ii) roots colonization in anoxia is in progress.

2 Experimental procedures

2.1 Bacterial strains and growth conditions

The strains used in this study are displayed in supplementary Table S1. *A. fabrum* C58 and *E. coli* were grown individually on YPG (Yeast extract, 5 g/L; Peptone, 5 g/L; Glucose, 10 g/L) and TY (Bactotryptone, 5 g/L; Yeast extract, 3 g/L) medium at 28°C and 37°C, respectively. When required, media were supplemented with antibiotics at the following concentrations: neomycin, 25 µg/L; kanamycin, 25 µg/L. Media were solidified with 15 g/L agar. The characteristics of the 4 replicons of *A. fabrum* C58 genome are summarized in Table 1 according to Wood et al. (2001).

Table 1. Characteristics of the 4 replicons of *A. fabrum* C58 genome (from Wood et al., 2001).

| Feature | Circular | Linear | pAT | pTi | Total |
|-----------------------------|-----------|-----------|---------|---------|--------------|
| Size (bp) | 2,841,490 | 2,075,560 | 542,779 | 214,233 | 5,674,062 |
| G+C content (%) | 59.4 | 59.3 | 57.3 | 56.7 | 58.13 |
| Protein-coding genes | | | | | |
| Assigned function | 1715 | 1286 | 333 | 141 | 3475 (64.1%) |
| Conserved hypothetical | 710 | 353 | 128 | 45 | 1236 (22.8%) |
| Hypothetical | 364 | 243 | 89 | 12 | 708 (13.1%) |
| Total | 2789 | 1882 | 550 | 198 | 5419 |
| Average ORF size (bp) | 892 | 988 | 843 | 925 | 922 |
| Coding (%) | 87.9 | 89.9 | 85.4 | 85.5 | 88.3 |
| Regulators (%) | 7.7 | 10.4 | 11.8 | 5.1 | 9.0 |
| ABC transport | 47 | 80 | 20 | 6 | 153 |
| RNA | | | | | |
| rRNA | 2 | 2 | 0 | 0 | 4 |

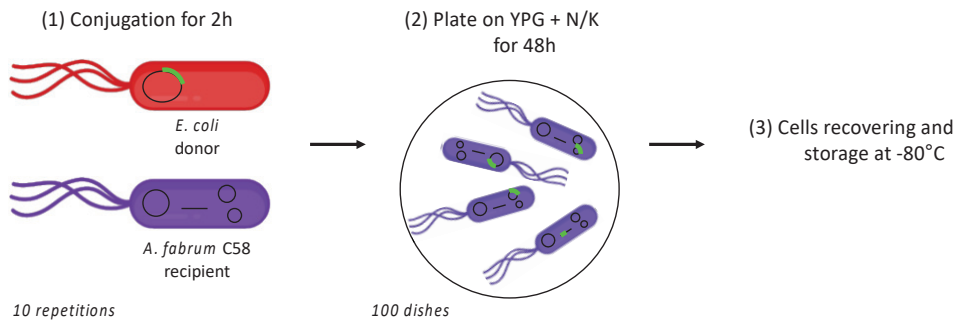
| | | | | | |
|---------------|----|----|----|---|----|
| tRNA | 40 | 13 | 0 | 0 | 53 |
| tmRNA | 1 | 0 | 0 | 0 | 1 |
| IS elements | 2 | 10 | 10 | 2 | 24 |
| Phage-related | 10 | 1 | 1 | 0 | 12 |

2.2 Construction of the transposon library and utilization

A. fabrum C8 was mutagenized using a mariner transposon carrying a neomycin resistance cassette conferring resistance to both neomycin and kanamycin (Fig 1a). *A. fabrum* C58 recipient and *E. coli* donor were cultivated separately on YPG and TY supplemented with 300 μ M diaminopimelic acid (DAP) respectively. Cells of *A. fabrum* C58 and *E. coli* corresponding to 50 OD_{600 nm} units of overnight cultures were mixed and centrifuge for 30 min at 5500 rpm. The cell mixture was resuspended in 1 mL TY medium and deposited in 10 drops on TY agar medium supplemented with DAP. After 2 h, bacteria were removed from the plate and resuspended in 10 mL YPG. Serial dilutions (to 10⁻⁸) and plating were performed to estimate the efficiency of mutagenesis and determine the number of mutants. The remaining culture was serial diluted to 10⁻³ and plated on YPG agar supplemented with neomycin and kanamycin for 48 hours. The resulting colonies were collected, aliquoted in YPG with 25 % (v/v) glycerol and conserved at - 80°C.

For anoxic and plants experiments, 100 μ L of the library were thawed slowly on ice and cultured in liquid YPG medium (4h at 28°C) to revive the cells. Bacteria were washed twice with 0.8% NaCl solution and used to inoculate (i) YPG medium supplemented with nitrate or nitrite under anoxic condition and (ii) roots of maize plants under anoxic condition at 2.10⁵ CFU/mL (Fig. 2). Two repetitions were performed for each experiment. After experiments, bacterial cells were centrifuged and stored at - 20°C for further DNA manipulation.

a) Transposon mutant library construction



b) DNA library preparation for high-throughput sequencing

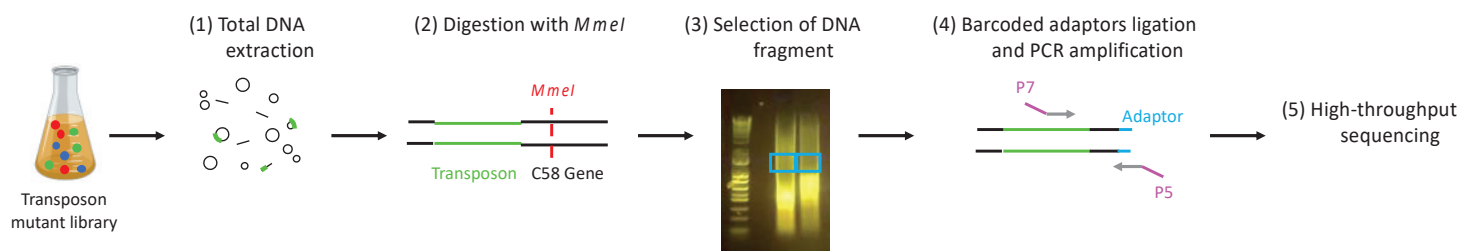


Figure 1. Tn-seq library preparation. (a) Mutant library construction. The plasmid containing the transposon was transferred (1) from *E. coli* to *A. fabrum* C58 by conjugation. To minimize replication and the occurrence of sibling mutants, we opted for a short time of conjugation to 2h at 28°C. Ten conjugations were performed individually. The *A. fabrum* C58 transconjugants were (2) plate on YPG with kanamycin (25 µg/mL) and neomycin (25 µg/mL) and grown at 28°C for 48h, the necessary time to see colonies. The cells were then (3) recovered and stored at -80°C. **(b)** DNA library preparation for high-throughput sequencing. Total DNA were (1) extracted from 2 aliquots of the mutant library. Total DNA were (2) digested with *MmeI*. After gel migration, (3) DNA containing transposon sequence and 16 nucleotides of the adjacent gene corresponding to 1.3 kb to 1.8 kb were recovered. These DNA sequences were (4) ligated with a unique adaptor and amplified by PCR with specific primers containing sequences for (5) Illumina sequencing.

2.3 In vitro experiment under anaerobic condition

4.10⁵ mutant cells of the transposon library were grown under anoxic or oxic condition (control) on YPG supplemented with either nitrate or nitrite at 28°C without shaking in 160 mL flasks closed hermetically with corks (Fig. 2a). 140 mL of helium were injected into flasks to obtain anoxic condition. 25 flasks were used for each condition and experiments were performed in duplicates. NO₃⁻ was added at 20 mM and bacterial cells were grown for 48 h. NO₂⁻ was added at 2 mM and bacterial cells were grown for 7 days. After growth, bacterial cells were centrifuged and stored at -80°C.

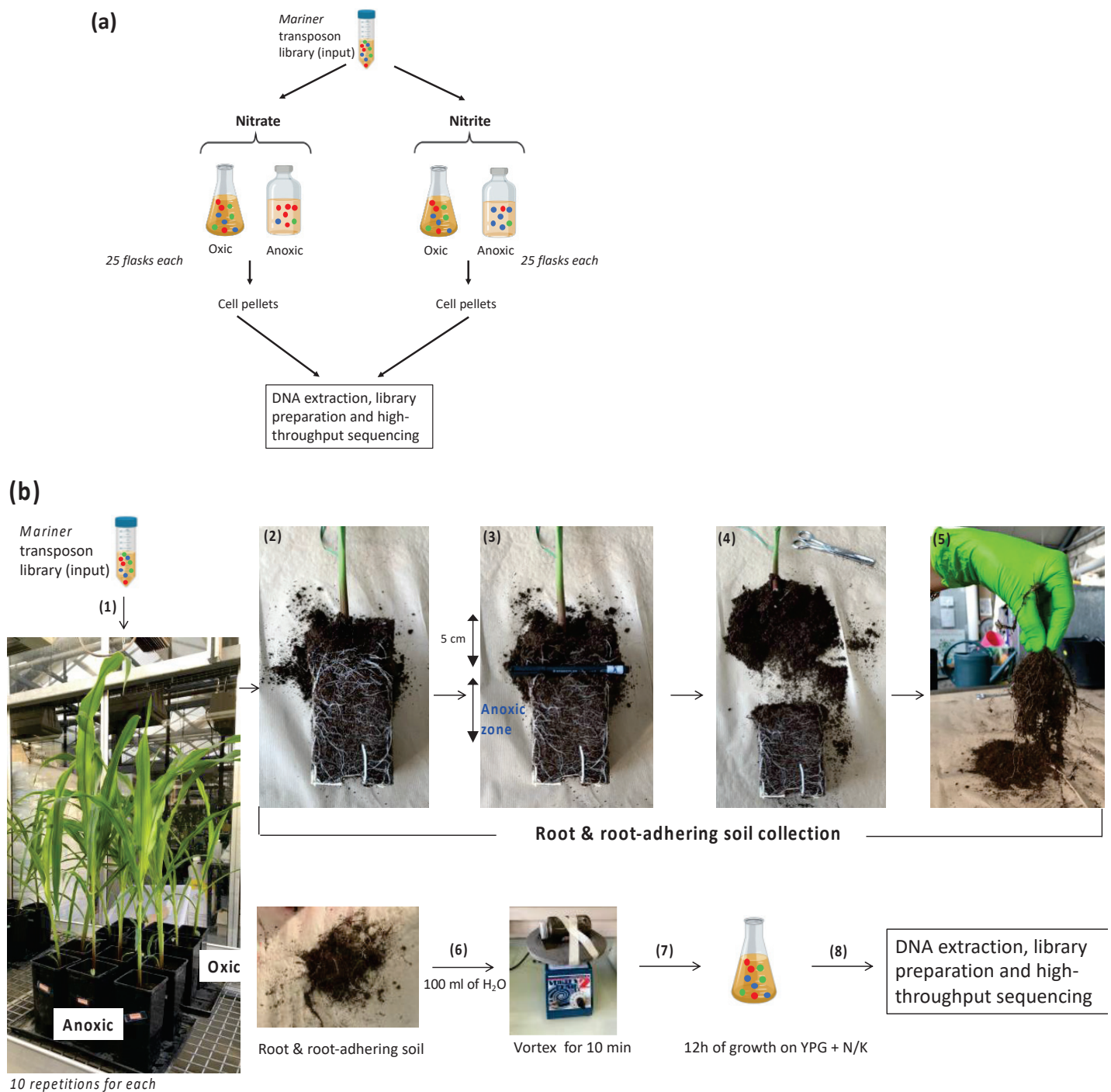


Figure 2. Set-up of the Tn-seq experiment *in vitro* and *in planta*. (a) The transposon insertion library was added to YPG medium supplemented with either 20 mM of nitrate or 2 mM of nitrite under anoxic or oxic condition. (b) The transposon insertion library was inoculated (1) under anoxic or oxic condition to maize roots. After 2 weeks post inoculation, (2) the root system and the root-adhering soil (RAS) from plants growing under oxic and anoxic conditions were harvested. (3 and 4) The 5 first centimeters from the top of the pots were removed. The rest of the roots with the RAS of each plant were vortexed for 10 min after adding 100 ml of water (6). After sedimentation, 10 ml of water containing bacteria from each root system were inoculated to YPG medium supplemented with neomycin (N) and kanamycin (K) and grown for 12 h (6 and 7). Bacterial cells were centrifugated and DNA was extracted from each pellet for library preparation and high-throughput sequencing (8).

2.4 *In planta* experiment under anaerobic condition

Maize seeds were planted in pots containing 600 g of compost (Klasmann Deilmann) amended with nitrate and incubated in a greenhouse (13 h day at 22°C/11 h night at 18°C), with an approximate light intensity of 8–10 klux for 3 weeks. 10 plants were used for each condition (oxic and anoxic) and experiments were performed in duplicates (Fig. 2b). Each plant was inoculated with $5 \cdot 10^5$ mutant cells of the transposon library diluted in 50 ml sterilized water. To optimize cells root colonization, anoxic condition was applied 2 days post inoculation. To mimic anoxic condition found in soil, 20 plants (10 per replicate) were flooded by adding 200 mL water every day. The 20 oxic plants were watered for normal growth. Anoxic and oxic conditions were verified using O₂ sensor during plant growth. 2 weeks post inoculation, plants were harvested. To recover root system and root-adhering soil (RAS) from plants growing under oxic and anoxic conditions, the 5 first centimeters from the top of the pots were removed (Fig. 2b). The rest of the roots with the RAS of each plant were vortexed for 10 min after adding 100 ml water. After 30 min of sedimentation, 10 ml water containing bacteria from each root system were inoculated to YPG medium supplemented with neomycin and kanamycin and grown for 12 h. Bacterial cells were centrifugated and pellets were stored at -80°C.

2.5 DNA isolation and library preparation for high-throughput sequencing

High-throughput sequencing were performed in duplicates for (i) the initial library, (ii) YPG medium under oxic condition with either nitrate or nitrite, (iii) YPG medium under anoxic condition with either nitrate or nitrite, (iv) plants under oxic condition and (v) plants under anoxic condition. Sequencing libraries were prepared by extracting genomic DNA (50 µg) using a Promega Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA), digesting it with MmeI as previously describe by Royet *et al.* (2018), purifying it (QIAquick PCR Purification Kit, Qiagen, Germany) and concentrating it (Fig 1b). Digested DNA samples were run on a 1% agarose gel, the 1.3-1.8 kb band containing the transposon and adjacent DNA were cut out and DNA was extracted from the gel using QIAquick Gel Extraction Kit (Qiagen, Germany). Barcoded adaptors were ligated with DNA of each sample using T4 DNA ligase (Biolabs, Ipswich, MA, USA). The adaptors contained a five-nucleotide long barcode that is unique for each experiment (Royet *et al.*, 2018). The transposon adjacent DNA were amplified by polymerase chain reactions (PCRs) using the ligation product as template. Each reaction contained specific primers which annealed to the P7 Illumina sequence of the transposon and the P5 of the adaptor. Samples were concentrated and checked by agarose gel migration. The 125 pb band were extracted and purified as described above. DNA samples were finally dialyzed on MF-Millipore™ Membrane Filters for 4 h. Quality controls of the Tn-seq DNA libraries (size of the fragments and concentration) were

performed at Biofidal (Vaulx-en-Velin, France). Tn-seq DNA libraries were sequenced with HiSeq 2500 technology (Illumina, San Diego, CA, USA) at MGX (CNRS Sequencing Service, Montpellier, France).

2.6 Bioinformatic analyses

Raw reads were filtered with cutadapt v1.15 (Martin, 2011) to retain only the reads containing the mariner inverted left repeat (ACAGGTTGGATGATAAGTCCCCGGTCTT) and trimmed to remove it. Then, the resulting fastQ files were converted in fasta format and normalized by randomly subsampling each sample to the lowest sequencing yield with seqtk v1.2 (Shen et al., 2016). Trimmed reads were then analyzed and mapped to the genome using a modified version of Transit Pre-Processor tool (TPP) (DeJesus et al., 2015) as further described in Royet et al. (2018). Eventually, conditional essentiality of genes was determined with TRANSIT v2.3.4 (DeJesus et al., 2015) using the HMM method (DeJesus & Iøerger, 2013). Parameters were set to ignore insertions in the 10% N and C-terminal regions or the ORFs which could be mutagenized without disrupting the gene function, management of duplicates was performed by using the mean read count among them and TTR normalization and LOESS correction for genome positional bias were applied. KOGG functional annotations were retrieved from MAGE database (Vallenet et al., 2017).

3 Results and discussion

3.1 Genome-wide mutant library of *A. fabrum* C58

3.1.1 Characterization of *A. fabrum* C58 transposon library

For the construction of the Tn-seq library, we used a *Himar9* mariner transposon derivative carrying *MmeI* site in its extremities and a kanamycin resistance cassette (Wiles et al., 2013). We obtained approximately 5.10^{10} mutant cells. To validate the quality of the Tn-seq library, two DNA libraries were prepared from two aliquots and subjected to high-throughput sequencing. The *A. fabrum* C58 genome contained 115,525 TA sites positioned along the circular chromosome (55,348 TA within 2,841,580 bp), linear chromosome (41,503 TA within 2,075,577 bp), and the two plasmids, the pAt (13,084 TA within 542,868 bp) and pTi (5,590 TA within 214,233 bp) that are potential insertion sites of the *Himar9* transposon (Gonzalez-Mula et al., 2019). TPP software (DeJesus et al., 2015) was used to determine the number of reads at each TA site. Number of TA containing insertions corresponded to 77 % of the total TA sites in the circular chromosome, 85% in the linear chromosome, 88% in the Ti plasmid and 89% in the AT plasmid. These results suggested a high saturation of our library as explained previously by DeJesus et al. (2017) using *Mycobacterium tuberculosis* strain. Indeed, the authors considered that with a level of coverage about 84.3% of the potential TA insertions sites, the saturation of their library was optimal. The transposon was inserted homogeneously among C58 genome (Fig. 3a, b and Fig. S1). The mean number of reads over non-zero TA is 169, 146, 135 and 140 reads for the circular

chromosome, linear chromosome, pAT and pTi, respectively. This corresponds to a total of 10 millions of reads. The results were reproducible among duplicates with a Pearson correlation coefficient about 0,98 for the circular and for the linear chromosome (Fig. 3c, d). After analyses, 100,000 independent mutants were obtained. These results attest to the high quality of the Tn-seq library.

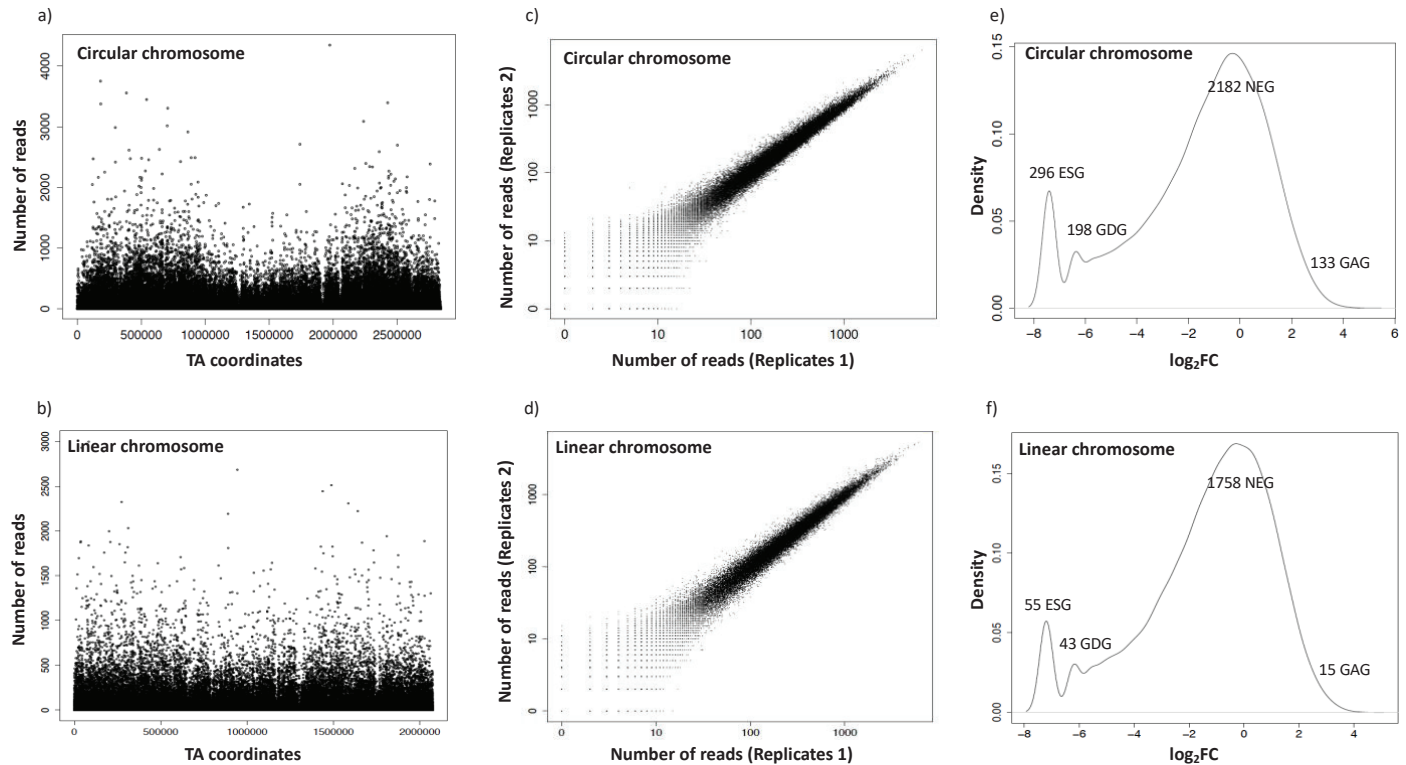


Figure 3. Quality control of the transposon sequencing (Tn-seq) *Agrobacterium fabrum* C58 libraries. Frequency and distribution of transposon sequence reads along the (a) circular chromosome and (b) the linear chromosome. The localization of transposon insertions was homogeneous along the linear chromosome, whereas, spots with less insertions were noticeable for the circular chromosome. Biological reproducibility of the results for (c) the circular chromosome and (d) the linear chromosome. Analysis of DNA samples corresponded to two independent cultures of two independent mutant library. The calculated Pearson correlation coefficients were 0,98 for each replicon. Density plot of log₂ fold change (log₂FC; measured reads/expected reads per gene) for (e) the circular chromosome and (f) the linear chromosome. ESG for essential genes, GDG for growth defect genes, GAG for growth advantage genes and NEG for non-essential genes.

3.1.2 Essential genes in *A. fabrum* C58

To determine essential genes in *A. fabrum* C58, we calculated, using TRANSIT software (DeJesus et al., 2015), for each gene a log₂ fold change (log₂FC) corresponding to the ratio between the measured and expected number of reads. We opted for the Hidden Markov Model (HMM) which predicts the category of the gene between essential, growth defect, growth advantage and non-essential. Moreover, the high saturation of the library combined to the HMM will enabled us to identify small ORFs that are essential for growth (DeJesus et al., 2017). Essential genomics regions were determined as regions without transposon insertions at TA sites. Remarkably, essential genes were only found in linear and circular chromosomes (Table S3) and one exception on pTi encoding a hypothetical protein non-conserved among agrobacteria. Essential genes (ESG) were distributed

homogeneously and spread along the circular chromosome (Fig. 4a) attesting the low growth rate of this strain compared to *E. coli*, a fast-growing strain where essential genes are organized around the replicative origin (Gerdes et al., 2003). Interestingly, no essential genes were evidenced in two regions, from Atu0795 to Atu0968 (encoding some ABC transporters, Na⁺/H⁺ antiporter and hypothetical proteins) and from Atu2251 to Atu2420 (encoding several ABC transporters and hypothetical proteins). In addition, these two regions were (i) well conserved in all *A. tumefaciens* complex species, (ii) presented the same GC% than the rest of the genome and (iii) devoid from natural transposon. These findings disproved a recent acquisition of these regions by *A. fabrum* C58. Intriguingly, these two regions are symmetric according to segment orientations toward the origin-terminus axis. Furthermore, they contain more genes conferring a growth advantage when disrupted than the rest of the circular chromosome (6.8% and 20% respectively against 4.4%) and thus were more permissive to transposon insertion. Collectively, these results suggested that these regions were recombination hotspots that may give adaptative advantage in certain conditions.

The circular chromosome harbor 296 ESG whereas the linear harbor 55 ESG representing 10 and 3 % of their related replicon, respectively. These genes take part in essential cellular pathways, such as translation, metabolism, cell membrane biogenesis (Table S3 and Fig. 4). In agreement with Curtis and Brun (2014), genes identified as essential for developmental and cell cycle systems were already identified as essential among alphaproteobacteria, such as *Brevundimonas subvibrioides*, *Caulobacter crescentus* and *A. tumefaciens*. As mention above, we obtained 77 and 85 % of TA insertions in circular and linear chromosome, respectively. Notably, 23 and 15 % of TA sites present no insertion in circular and linear chromosome, respectively. 8.7 and 2.6 % are justified by the gene essentiality and the remaining percent could be explained by nonpermissive TA sites for insertion as also suggested in previous study by Dejesus et al. (2017).

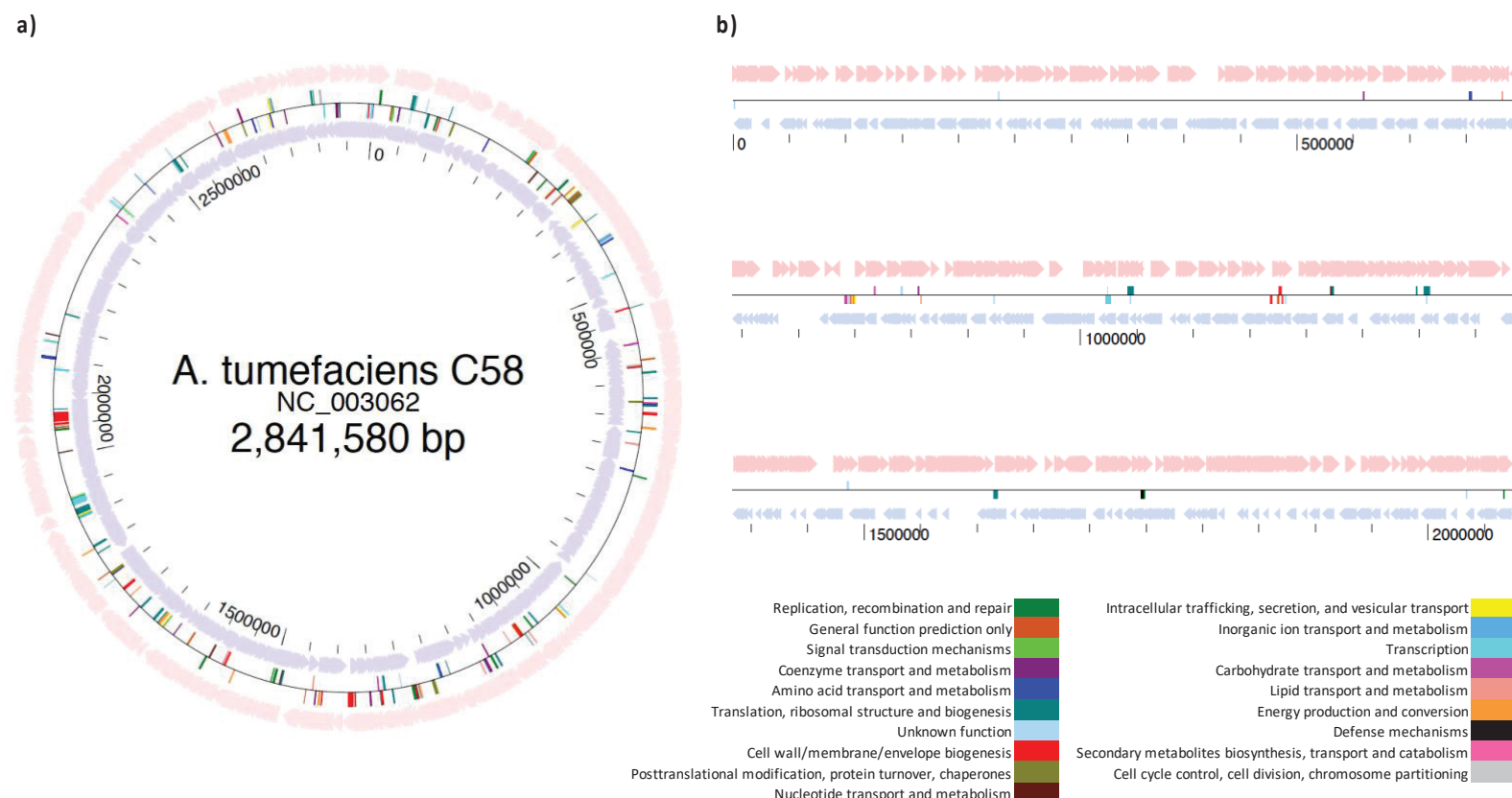


Figure 4. Distribution and function of essential genes. a) Essential genes on the circular chromosome. b) Essential genes on the linear chromosome. ESG are representing by different colors according to their involvement in different pathways. Genes on the positive and negative strands are represented by red and blue arrows respectively. Essential genes are figured by red and blue bar. Linear chromosome is composed of 3 lines for esthetical reasons. Representation were obtained from Lasergene Genvision software.

In addition, circular chromosome harbor 198 genes categorized as growth defect genes (GDG, i.e. loss of fitness due to transposon insertion), 133 as growth advantage genes (GAG, i.e. gain of fitness due to transposon insertion), and 2182 as non-essential genes (NEG, i.e. no difference of fitness) (Fig. 3e). For the linear chromosome, 43 genes were categorized as GDG, 15 as GAG and 1758 as NEG (Fig. 3f). Interestingly, genes conferring a growth advantage when disrupted represented 11.75 % of total genes of the pTi plasmid, whereas, they represented only 4.4 and 0.8 % of the total genes of the circular and linear chromosome, respectively. These genes are known to deal with the virulence (type IV secretion system and conjugal transfer), the use of nopaline (a carbon source specific of the tumor environment) and ABC transporters *in vivo*. Even if, these genes are involved in plant niche-construction (Gonzalez-Mula et al., 2019) and confer a competitive advantage for *Agrobacteria in planta* (Dessaux & Faure, 2018), they have high metabolic costs *in vitro*. Collectively, these findings illustrate that Ti plasmid due to its metabolic costs can be considered as genetic burden which explain its presence only in certain agrobacteria and its acquisition by avirulent one only during plant infection (Dessaux & Faure, 2018).

3.2 A Tn-Seq screen will identify *A. fabrum* C58 fitness determinants under denitrification condition *in vitro* and *in planta*

3.2.1 *A. fabrum* C58 key-genes for respiring nitrate and nitrite

To identify genes required for *A. fabrum* C58 anaerobic respiration, we opted for a transposon mutagenesis screen on nitrate and nitrite followed by high sequencing (Tn-seq) strategy. We chose nitrate as TEA to reveal regulators and transporters involved in nitrate reduction, the first step of denitrification. As nitrate reduction is the most energetic step that sustain substantial bacterial growth (Thauer et al., 1989), genes involved in the next steps will probably not be evidenced. Indeed, recently, Tn-seq experiment on *Pseudomonas brassicacearum* NFM421 strain growing under anoxic condition with nitrate as TEAs revealed almost genes involved in nitrate reduction (W. Achouak, personal communication). To overcome this limitation, we decided to perform Tn-seq experiment on nitrite, the substrate of the second step of denitrification. Thus, we hope to evidence regulators and transporters involved in nitrite (2nd step of denitrification) and also in NO reduction (3rd step of denitrification). Another limitation which may occur was previously characterized as complementation in *trans*, whereby a transposon mutant's phenotype is suppressed by bacteria WT for the locus present in the transposon library (Carey et al., 2018). The authors in this publication believe that these effects are limited to secreted products. We trust that our experimental design will allows us to obtain a picture of regulators and transporters involved in *A. fabrum* C58 denitrification *in vitro*. The revelation of genes encoding for denitrification reductases, such as *napA*, *nirK*, *norC* (Baek & Shapleigh, 2005; Lecomte et al., chapter 1) will be a good indication that we targeted all denitrification steps performed by *A. fabrum* C58.

The constructed mutant library was inoculated *in vitro* on YPG (rich medium). As growth under anoxic condition is very slow on minimal medium, we used a rich medium to ensure the number of generations needed for Tn-seq experiment. In addition, no growth was observed on YPG without nitrogen oxides supplementation under anoxic condition.

In order to reveal denitrification regulators under anoxic condition, we used as control the mutant library grown on YPG supplemented with nitrate or nitrite under oxic condition. We believe that growth under oxic condition is not the best control due to cell physiology, but no one is available. We think that Tn-seq analysis will reveal genes involved in denitrification pathways and also in cells maintenance, flagella establishment or essential genes involved in cell division, translation ... Among all identified genes, we will choose some of them that are particularly interesting to answer to our question. Then, mutants of these genes will be constructed, and their fitness will be evaluated during competition assay *in vitro* under anoxic condition with nitrate or nitrite as TEA.

3.2.2 Tn-seq reveals maize roots colonization genes of *A. fabrum* C58 under denitrification condition

To determine whether denitrification is a rhizosphere competence trait, we (i) identified denitrification genes required for *A. fabrum* C58 root colonization of maize rhizosphere under anoxic condition using Tn-seq experiment and (ii) will validate them by performing mutant competition assay *in vitro*.

We validated the denitrification condition in maize rhizosphere by measuring nitrate in soil and controlling anoxia in the course of plant growth. It is well known in complex environments, such as rhizosphere, in the presence of different TEAs, microorganisms generally prefer to use the most energetic one first. To date, denitrification and more precisely nitrate reduction step yields the highest energy to sustain bacterial growth in anoxia (Thauer et al., 1989; Lecomte et al., 2018). In our condition, nitrate was largely available in soil and anoxia was applied ensuring denitrification functioning. However, we cannot exclude the growth of certain mutants in denitrification pathway by respiring anaerobically certain root exudates as suggested in our previous work (Lecomte et al., chapter1). Indeed, *A. fabrum* C58 $\Delta napA$ mutant was able to colonize maize root and slightly growth anaerobically. We believe that denitrification genes will be found in growth defect genes category.

One limitation of Tn-seq analysis during host colonization is the requirement for an outgrowth step after the recovery of bacteria from roots (Wilde et al., 2015). To avoid confounding effects on fitness calculations, the library was grown on YPG before maize inoculation and after roots recovery for a short time.

By comparing results obtained from anoxic condition *in planta* with those from anoxic condition *in vitro*, we expect to reveal denitrification genes required for *A. fabrum* C58 root colonization of maize rhizosphere under anoxic condition and hence determine whether denitrification is a rhizosphere competence trait. To validate candidate genes, mutants will be constructed, and their fitness will be evaluated using competition assay as previously described by Lecomte et al. (chapter 1).

Supplementary data

Table S1. Bacterial strains and plasmid used in this study.

| Strains | Relevant genotype and description | Reference or source |
|------------------------------------|---|------------------------|
| <i>Escherichia coli</i> K12 | | |
| MFDpir | RP4-2-Tc::(Δ Mu1::aac(3)IV- Δ aphA- Δ nic35- Δ Mu2::zeo) Δ dapA::erm-pir) Δ recA | Ferrières et al., 2010 |
| <i>Agrobacterium fabrum</i> | | |
| C58 WT | <i>Agrobacterium fabrum</i> C58 wild type | CFBP 1903 |
| Plasmid | Description | |
| pSAM-Ec | Suicide mobilizable vector; Amp ^R , Km ^R resistance gene bordered by mariner inverted repeat sequence containing Mmel restriction site, himar1-C9 transposase gene under the control of <i>P</i> _{lac} . | Wiles et al., 2013 |

Table S2. Oligonucleotides used in this study.

| Oligonucleotide | Sequence | Reference |
|-----------------|---|----------------------|
| LIB_AdaptT | TTCCCTACACGACGCTCTTCCGATCTNN | Skurnik et al., 2013 |
| LIB_AdaptB | AGATCGGAAGAGCGTCGTGTAGGGAA | Skurnik et al., 2013 |
| LIB_PCR_5 | CAAGCAGAAGACGGCATACGAAGACCGGGGACTTATCATCCAACCTGT | Skurnik et al., 2013 |
| LIB_PCR_3 | AATGATACGGCGACCACCGAAGCTCTTCCCTACACGACGCTCTTCCGATCT | Skurnik et al., 2013 |

Table S3. Genes identified by transposon sequencing (Tn-seq) approach as essential. The accession number, the product of the gene, the function, the number of TAs in each one and the localization (circular or linear chromosome) are advise.

| Locus | Gene | Product | Function | nTA | Chromosome |
|---|--------|--|---|-----|------------|
| CELLULAR PROCESSES AND SIGNALING | | | | | |
| Atu0027 | - | two component sensor kinase | Signal transduction mechanisms | 38 | Circular |
| Atu0100 | kdsB | 3-deoxy-manno-octulosonate cytidyltransferase | Cell wall/membrane/envelope biogenesis | 16 | Circular |
| Atu0122 | dnaK | DNAK Protein | Posttranslational modification, protein turnover, chaperones | 24 | Circular |
| Atu0257 | - | hypothetical protein | Signal transduction mechanisms | 13 | Circular |
| Atu0342 | lspA | lipoprotein signal peptidase | Cell wall/membrane/envelope biogenesis | 13 | Circular |
| Atu0346 | glnD | uridylyltransferase/uridylyl-removing enzyme UTase | Posttranslational modification, protein turnover, chaperones | 43 | Circular |
| Atu0351 | - | hypothetical protein | Posttranslational modification, protein turnover, chaperones | 6 | Circular |
| Atu0352 | - | hypothetical protein | Posttranslational modification, protein turnover, chaperones | 6 | Circular |
| Atu0384 | vidC | preprotein translocase 60 kD membrane subunit | Intracellular trafficking, secretion, and vesicular transport | 34 | Circular |
| Atu0539 | c1 | bacteriophage repressor protein C1 | Cell wall/membrane/envelope biogenesis | 8 | Circular |
| Atu0682 | hisS-1 | histidyl-tRNA synthetase | Posttranslational modification, protein turnover, chaperones | 27 | Circular |
| Atu0683 | groEL | GroEL chaperonin | Posttranslational modification, protein turnover, chaperones | 20 | Circular |
| Atu0695 | ileS | isoleucyl-tRNA synthetase | Cell wall/membrane/envelope biogenesis | 41 | Circular |
| Atu0697 | kdtA | 3-deoxy-D-manno-octulosonic acid transferase | Cell wall/membrane/envelope biogenesis | 24 | Circular |
| Atu1034 | - | DNA polymerase | Intracellular trafficking, secretion, and vesicular transport | 7 | Circular |
| Atu1105 | - | hypothetical protein | Posttranslational modification, protein turnover, chaperones | 12 | Circular |
| Atu1106 | gmk | guanylate kinase | Cell wall/membrane/envelope biogenesis | 12 | Circular |
| Atu1259 | argC | N-acetyl-gamma-glutamyl-phosphate reductase | Posttranslational modification, protein turnover, chaperones | 15 | Circular |
| Atu1261 | rpsI | 30S ribosomal protein S9 | Posttranslational modification, protein turnover, chaperones | 11 | Circular |
| Atu1289 | nuoN | NADH ubiquinone oxidoreductase chain N | Cell wall/membrane/envelope biogenesis | 25 | Circular |
| Atu1290 | birA | birA bifunctional protein | Defense mechanisms | 14 | Circular |
| Atu1341 | - | hypothetical protein | Cell wall/membrane/envelope biogenesis | 8 | Circular |
| Atu1381 | pyrH | uridylylate kinase | Cell wall/membrane/envelope biogenesis | 13 | Circular |

| | | | | | |
|---------|-------|--|---|----|----------|
| Atu1382 | rff | ribosome recycling factor | Cell wall/membrane/envelope biogenesis | 7 | Circular |
| Atu1384 | cdsA | phosphatidate cytidyltransferase | Cell wall/membrane/envelope biogenesis | 18 | Circular |
| Atu1386 | lpxD | UDP glucosamine N-acyltransferase | Cell wall/membrane/envelope biogenesis | 22 | Circular |
| Atu1424 | fabZ | Dehydratase | Cell wall/membrane/envelope biogenesis | 5 | Circular |
| Atu1594 | gyrA | DNA gyrase subunit A | Cell wall/membrane/envelope biogenesis | 40 | Circular |
| Atu1704 | gyrB | DNA gyrase subunit B | Intracellular trafficking, secretion, and vesicular transport | 28 | Circular |
| Atu1705 | nadE | NAD (+) synthetase | Intracellular trafficking, secretion, and vesicular transport | 33 | Circular |
| Atu1706 | coaD | phosphopantetheine adenylyltransferase | Intracellular trafficking, secretion, and vesicular transport | 7 | Circular |
| Atu1786 | valS | valyl-tRNA synthetase | Cell wall/membrane/envelope biogenesis | 36 | Circular |
| Atu1787 | thrS | threonyl-tRNA synthetase | Cell wall/membrane/envelope biogenesis | 36 | Circular |
| Atu1822 | glmU | UDP-N-acetylglucosamine pyrophosphorylase | Posttranslational modification, protein turnover, chaperones | 29 | Circular |
| Atu1823 | - | hypothetical protein | Posttranslational modification, protein turnover, chaperones | 5 | Circular |
| Atu1824 | - | tRNA-Arg | Posttranslational modification, protein turnover, chaperones | 4 | Circular |
| Atu1927 | rpoA | DNA-directed RNA polymerase alpha subunit | Intracellular trafficking, secretion, and vesicular transport | 17 | Circular |
| Atu1962 | rplJ | 50S ribosomal Protein L10 | Intracellular trafficking, secretion, and vesicular transport | 6 | Circular |
| Atu2086 | - | tRNA-Trp | Cell cycle control, cell division, chromosome partitioning | 5 | Circular |
| Atu2087 | thyA | thymidylate synthase | Cell cycle control, cell division, chromosome partitioning | 23 | Circular |
| Atu2088 | - | DNA ligase | Cell wall/membrane/envelope biogenesis | 42 | Circular |
| Atu2089 | - | hypothetical protein | Cell wall/membrane/envelope biogenesis | 21 | Circular |
| Atu2092 | ftsZ2 | cell division protein | Cell wall/membrane/envelope biogenesis | 19 | Circular |
| Atu2093 | ftsA | cell division protein | Cell wall/membrane/envelope biogenesis | 17 | Circular |
| Atu2094 | ftsQ | cell division protein | Cell wall/membrane/envelope biogenesis | 16 | Circular |
| Atu2095 | ddlB | D-alanylalanine synthetase | Cell cycle control, cell division, chromosome partitioning | 19 | Circular |
| Atu2096 | murB | UDP-N-acetylenolpyruvoylglucosamine reductase | Cell wall/membrane/envelope biogenesis | 16 | Circular |
| Atu2097 | murC | UDP-N-acetylmuramate-alanine ligase | Cell wall/membrane/envelope biogenesis | 17 | Circular |
| Atu2098 | murG | UDP-N-acetylglucosamine transferase | Cell wall/membrane/envelope biogenesis | 14 | Circular |
| Atu2099 | ftsW | cell division protein | Cell wall/membrane/envelope biogenesis | 15 | Circular |
| Atu2100 | murD | UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase | Cell wall/membrane/envelope biogenesis | 25 | Circular |
| Atu2102 | murF | UDP-MurNAc-pentapeptide synthetase+C54:C57 | Cell wall/membrane/envelope biogenesis | 18 | Circular |

| | | | | | |
|---------|------|--|---|----|----------|
| Atu2438 | - | chitooligosaccharide deacetylase | Signal transduction mechanisms | 23 | Circular |
| Atu2440 | - | hypothetical protein | Cell cycle control, cell division, chromosome partitioning | 8 | Circular |
| Atu2651 | atpD | ATP synthase beta chain | Posttranslational modification, protein turnover, chaperones | 23 | Circular |
| Atu2693 | gcp | O-sialoglycoprotein endopeptidase | Intracellular trafficking, secretion, and vesicular transport | 18 | Circular |
| Atu2697 | hem4 | Uroporphyrinogen III synthase HEM4 | Intracellular trafficking, secretion, and vesicular transport | 13 | Circular |
| Atu2759 | rplS | 50S ribosomal protein L19 | Cell cycle control, cell division, chromosome partitioning | 15 | Circular |
| Atu2829 | proB | gamma-glutamyl kinase | Cell cycle control, cell division, chromosome partitioning | 15 | Circular |
| Atu2830 | cgtA | GTP-binding protein | Cell wall/membrane/envelope biogenesis | 20 | Circular |
| Atu3711 | mesJ | hypothetical protein | Cell cycle control, cell division, chromosome partitioning | 23 | Linear |
| Atu3713 | palA | omp16 protein | Cell wall/membrane/envelope biogenesis | 9 | Linear |
| Atu3714 | tolB | translocation protein TolB | Intracellular trafficking, secretion, and vesicular transport | 22 | Linear |
| Atu3715 | tolA | hypothetical protein | Cell wall/membrane/envelope biogenesis | 10 | Linear |
| Atu3716 | tolR | tolR protein | Intracellular trafficking, secretion, and vesicular transport | 7 | Linear |
| Atu3717 | tolQ | tolQ protein | Intracellular trafficking, secretion, and vesicular transport | 15 | Linear |
| Atu3774 | kpsF | capsule expression protein | Cell wall/membrane/envelope biogenesis | 12 | Linear |
| Atu3924 | repA | replication protein A | Cell cycle control, cell division, chromosome partitioning | 27 | Linear |
| Atu4051 | exoO | succinoglycan biosynthesis glycosyltransferase | Cell wall/membrane/envelope biogenesis | 22 | Linear |
| Atu4052 | exoM | UDP-hexose transferase | Cell wall/membrane/envelope biogenesis | 15 | Linear |
| Atu4058 | exoW | succinoglycan biosynthesis protein | Cell wall/membrane/envelope biogenesis | 18 | Linear |
| Atu4060 | exoU | succinoglycan biosynthesis glycosyltransferase | Cell wall/membrane/envelope biogenesis | 20 | Linear |
| Atu4103 | cmk | cytidylate kinase | Cell wall/membrane/envelope biogenesis | 17 | Linear |
| Atu4600 | - | ABC transporter permease | Defense mechanisms | 40 | Linear |

INFORMATION STORAGE AND PROCESSING

| | | | | | |
|---------|------|---------------------------------------|---|----|----------|
| Atu0012 | gyrB | DNA gyrase subunit B | Replication, recombination and repair | 50 | Circular |
| Atu0082 | pnpA | polynucleotide nucleotidyltransferase | Translation, ribosomal structure and biogenesis | 26 | Circular |
| Atu0084 | rpsO | 30S ribosomal protein S15 | Translation, ribosomal structure and biogenesis | 5 | Circular |
| Atu0096 | - | DNA polymerase III%2C tau subunit | Replication, recombination and repair | 29 | Circular |
| Atu0112 | rpmF | 50S ribosomal protein L32 | Translation, ribosomal structure and biogenesis | 6 | Circular |

| | | | | | |
|---------|-------|--|---|----|----------|
| Atu0254 | infC | translation initiation factor 3 | Translation, ribosomal structure and biogenesis | 8 | Circular |
| Atu0258 | pheS | phenylalanyl-tRNA synthetase%2C alpha-subunit | Translation, ribosomal structure and biogenesis | 12 | Circular |
| Atu0259 | pheT | phenylalanyl-tRNA synthetase beta chain | Translation, ribosomal structure and biogenesis | 32 | Circular |
| Atu0301 | dnaN | DNA polymerase III%2C beta chain | Replication, recombination and repair | 14 | Circular |
| Atu0323 | rpsT | 30S ribosomal protein S20 | Translation, ribosomal structure and biogenesis | 5 | Circular |
| Atu0324 | dnaA | chromosomal replication initiator protein DnaA | Replication, recombination and repair | 25 | Circular |
| Atu0341 | trmH | rRNA methylase | Translation, ribosomal structure and biogenesis | 11 | Circular |
| Atu0349 | trpS | tryptophan-tRNA ligase | Translation, ribosomal structure and biogenesis | 18 | Circular |
| Atu0385 | rnpA | ribonuclease P protein component | Translation, ribosomal structure and biogenesis | 8 | Circular |
| Atu0483 | argF | ornithine carbamoyltransferase | Transcription | 10 | Circular |
| Atu0644 | ispB | octaprenyl-diphosphate synthase | Translation, ribosomal structure and biogenesis | 13 | Circular |
| Atu0676 | glyS | glycyl-tRNA synthetase%2C beta chain | Translation, ribosomal structure and biogenesis | 26 | Circular |
| Atu0686 | ribF | riboflavin biosynthesis bifunctional protein | Translation, ribosomal structure and biogenesis | 13 | Circular |
| Atu0724 | atpF | ATP synthase B chain | Translation, ribosomal structure and biogenesis | 3 | Circular |
| Atu0794 | thrC | threonine synthase | Replication, recombination and repair | 32 | Circular |
| Atu0987 | ccrM | cell cycle protein | Replication, recombination and repair | 18 | Circular |
| Atu1029 | - | hypothetical protein | Transcription | 4 | Circular |
| Atu1035 | rnpO | DNA-directed RNA polymerase omega subunit | Transcription | 5 | Circular |
| Atu1073 | rnc | ribonuclease III | Translation, ribosomal structure and biogenesis | 7 | Circular |
| Atu1084 | - | GTP-binding protein%2C Era family | Replication, recombination and repair | 7 | Circular |
| Atu1153 | - | hypothetical protein | Translation, ribosomal structure and biogenesis | 17 | Circular |
| Atu1158 | pgsA | phosphatidylglycerophosphate synthase | Replication, recombination and repair | 16 | Circular |
| Atu1172 | ribD* | bifunctional riboflavin deaminase-reductase | Transcription | 20 | Circular |
| Atu1246 | plsX | fatty acid/phospholipid synthesis protein | Translation, ribosomal structure and biogenesis | 16 | Circular |
| Atu1247 | - | tRNA-Pro | Translation, ribosomal structure and biogenesis | 2 | Circular |
| Atu1288 | lon | ATP-dependent protease LA | Translation, ribosomal structure and biogenesis | 34 | Circular |
| Atu1292 | proS | prolyl-tRNA synthetase | Replication, recombination and repair | 30 | Circular |
| Atu1324 | lolD | ABC transporter | Translation, ribosomal structure and biogenesis | 7 | Circular |
| Atu1339 | dnaE | DNA polymerase alpha chain | Translation, ribosomal structure and biogenesis | 75 | Circular |

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|---------|------|---|---|----|----------|
| Atu1342 | gatB | glutamyl-tRNA amidotransferase subunit B | Translation, ribosomal structure and biogenesis | 20 | Circular |
| Atu1377 | folP | dihydropteroate synthase | Translation, ribosomal structure and biogenesis | 12 | Circular |
| Atu1496 | - | hypothetical protein | Translation, ribosomal structure and biogenesis | 12 | Circular |
| Atu1497 | lipA | lipic Acid Synthetase | Replication, recombination and repair | 13 | Circular |
| Atu1507 | hfq | putative RNA-binding protein Hfq | Replication, recombination and repair | 4 | Circular |
| Atu1512 | metS | methionyl-tRNA synthetase | Replication, recombination and repair | 28 | Circular |
| Atu1622 | - | NADPH:quinone reductase | Replication, recombination and repair | 7 | Circular |
| Atu1703 | pyrG | CTP synthase | Translation, ribosomal structure and biogenesis | 35 | Circular |
| Atu1711 | tatC | SEC-independent protein translocase protein | Translation, ribosomal structure and biogenesis | 15 | Circular |
| Atu1719 | tatB | SEC-independent protein translocase protein | Translation, ribosomal structure and biogenesis | 2 | Circular |
| Atu1746 | tatA | SEC-independent protein translocase protein | Translation, ribosomal structure and biogenesis | 3 | Circular |
| Atu1860 | sufD | transport system | Translation, ribosomal structure and biogenesis | 16 | Circular |
| Atu1873 | sufB | ABC transporter subunit | Translation, ribosomal structure and biogenesis | 30 | Circular |
| Atu1922 | - | hypothetical protein | Translation, ribosomal structure and biogenesis | 11 | Circular |
| Atu1923 | rpsD | 30S ribosomal protein S4 | Transcription | 15 | Circular |
| Atu1924 | icdA | isocitrate dehydrogenase | Translation, ribosomal structure and biogenesis | 16 | Circular |
| Atu1925 | alaS | alanyl-tRNA synthetase | Translation, ribosomal structure and biogenesis | 39 | Circular |
| Atu1930 | rpsK | 30S ribosomal protein S11 | Translation, ribosomal structure and biogenesis | 2 | Circular |
| Atu1931 | rpsM | 30S ribosomal protein S13 | Translation, ribosomal structure and biogenesis | 9 | Circular |
| Atu1932 | adk | adenylate kinase | Translation, ribosomal structure and biogenesis | 10 | Circular |
| Atu1933 | rplA | Preprotein translocase SECY subunit | Translation, ribosomal structure and biogenesis | 34 | Circular |
| Atu1934 | rplR | 50S ribosomal protein L18 | Translation, ribosomal structure and biogenesis | 5 | Circular |
| Atu1935 | rplF | 50S ribosomal protein L6 | Translation, ribosomal structure and biogenesis | 19 | Circular |
| Atu1936 | rpsH | 30S ribosomal protein S8 | Translation, ribosomal structure and biogenesis | 9 | Circular |
| Atu1937 | rpsN | 30S ribosomal protein S14 | Translation, ribosomal structure and biogenesis | 7 | Circular |
| Atu1938 | rplE | 50S ribosomal protein L5 | Translation, ribosomal structure and biogenesis | 12 | Circular |
| Atu1939 | rplX | 50S ribosomal protein L24 | Translation, ribosomal structure and biogenesis | 6 | Circular |
| Atu1940 | rplN | 50S ribosomal protein L14 | Translation, ribosomal structure and biogenesis | 9 | Circular |
| Atu1941 | rpsQ | 30S ribosomal protein S17 | Translation, ribosomal structure and biogenesis | 6 | Circular |

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|---------|------|---|---|----|----------|
| Atu1942 | rpmC | 50S ribosomal protein L29 | Translation, ribosomal structure and biogenesis | 2 | Circular |
| Atu1943 | rplP | 50S ribosomal protein L16 | Translation, ribosomal structure and biogenesis | 13 | Circular |
| Atu1944 | rpsC | 30S ribosomal protein S3 | Translation, ribosomal structure and biogenesis | 10 | Circular |
| Atu1945 | rplV | 50S ribosomal protein L22 | Translation, ribosomal structure and biogenesis | 5 | Circular |
| Atu1946 | rpsS | 30S ribosomal protein S19 | Translation, ribosomal structure and biogenesis | 9 | Circular |
| Atu1947 | rplB | 50S ribosomal protein L2 | Translation, ribosomal structure and biogenesis | 22 | Circular |
| Atu1955 | rplW | 50S ribosomal protein L23 | Transcription | 10 | Circular |
| Atu1956 | rplD | 50S ribosomal protein L4 | Transcription | 7 | Circular |
| Atu1957 | rplC | 50S ribosomal protein L3 | Translation, ribosomal structure and biogenesis | 18 | Circular |
| Atu1958 | rpsJ | 30S ribosomal protein S10 | Translation, ribosomal structure and biogenesis | 7 | Circular |
| Atu1959 | rpoC | DNA-directed RNA polymerase beta' chain | Translation, ribosomal structure and biogenesis | 69 | Circular |
| Atu1960 | rpoB | DNA-directed RNA polymerase beta chain | Translation, ribosomal structure and biogenesis | 71 | Circular |
| Atu1961 | rplL | 50S ribosomal protein L7/L12 | Transcription | 6 | Circular |
| Atu2082 | nusG | transcription antitermination protein NusG | Replication, recombination and repair | 9 | Circular |
| Atu2167 | - | hypothetical protein | Transcription | 7 | Circular |
| Atu2205 | - | hypothetical protein | Transcription | 5 | Circular |
| Atu2206 | rnpB | RNAase P RNA | Transcription | 8 | Circular |
| Atu2250 | carB | carbamoylphosphate synthase large subunit | Translation, ribosomal structure and biogenesis | 47 | Circular |
| Atu2434 | papS | poly(A) polymerase | Transcription | 14 | Circular |
| Atu2553 | - | hypothetical protein | Translation, ribosomal structure and biogenesis | 9 | Circular |
| Atu2699 | - | hypothetical protein | Translation, ribosomal structure and biogenesis | 15 | Circular |
| Atu2703 | fstY | cell division particle | Translation, ribosomal structure and biogenesis | 20 | Circular |
| Atu2748 | ffh | signal recognition particle protein | Translation, ribosomal structure and biogenesis | 16 | Circular |
| Atu2750 | rpsP | 30S ribosomal protein S16 | Replication, recombination and repair | 3 | Circular |
| Atu2763 | - | hypothetical protein | Transcription | 9 | Circular |
| Atu2784 | mtrA | two component response regulator | Translation, ribosomal structure and biogenesis | 17 | Circular |
| Atu2785 | nadD | nicotinic acid mononucleotide adenylyltransferase | Translation, ribosomal structure and biogenesis | 8 | Circular |
| Atu2828 | proA | gamma-glutamyl phosphate reductase | Transcription | 17 | Circular |
| Atu2833 | rpmA | 50S ribosomal protein L27 | Transcription | 8 | Circular |

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|---------|------|------------------------------------|---|----|----------|
| Atu8066 | gidB | glucose inhibited division protein | Translation, ribosomal structure and biogenesis | 22 | Circular |
| Atu3923 | repB | replication protein B | Transcription | 17 | Linear |
| Atu4064 | rpsU | 30S ribosomal protein S21 | Translation, ribosomal structure and biogenesis | 6 | Linear |
| Atu4104 | rpsA | 30S ribosomal protein S1 | Translation, ribosomal structure and biogenesis | 18 | Linear |
| Atu4176 | - | hypothetical protein | Translation, ribosomal structure and biogenesis | 17 | Linear |
| Atu4473 | lysS | lysyl-tRNA synthetase | Translation, ribosomal structure and biogenesis | 18 | Linear |
| Atu4474 | gltX | glutamyl-tRNA synthetase | Translation, ribosomal structure and biogenesis | 17 | Linear |
| Atu4601 | - | IS426 transposase | Replication, recombination and repair | 15 | Linear |
| Atu4602 | - | IS426 transposase | Replication, recombination and repair | 6 | Linear |
| Atu4890 | - | IS426 transposase | Replication, recombination and repair | 15 | Linear |
| Atu4891 | - | IS 426 transposase | Replication, recombination and repair | 6 | Linear |

METABOLISM

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|---------|-------|--|--|----|----------|
| Atu0037 | panK | pantothenate kinase | Coenzyme transport and metabolism | 19 | Circular |
| Atu0038 | hisE | phosphoribosyl-ATP pyrophosphohydrolase | Amino acid transport and metabolism | 2 | Circular |
| Atu0183 | dapB | 4-hydroxy-tetrahydrodipicolinate reductase | Amino acid transport and metabolism | 9 | Circular |
| Atu0281 | guaA | GMP synthase | Nucleotide transport and metabolism | 29 | Circular |
| Atu0320 | ubiE | biosynthesis methyltransferase | Coenzyme transport and metabolism | 21 | Circular |
| Atu0421 | - | tRNA-Arg | Inorganic ion transport and metabolism | 3 | Circular |
| Atu0422 | pstC | ABC transporter protein | Inorganic ion transport and metabolism | 22 | Circular |
| Atu0423 | pstA | ABC transporter protein | Inorganic ion transport and metabolism | 18 | Circular |
| Atu0428 | - | hypothetical protein | Amino acid transport and metabolism | 5 | Circular |
| Atu0429 | argD | acetylornithine aminotransferase | Amino acid transport and metabolism | 12 | Circular |
| Atu0600 | - | tRNA-Thr | Carbohydrate transport and metabolism | 5 | Circular |
| Atu0624 | - | hypothetical protein | Nucleotide transport and metabolism | 2 | Circular |
| Atu0632 | guaB | inosine-5'-monophosphate dehydrogenase | Lipid transport and metabolism | 22 | Circular |
| Atu0634 | - | hypothetical protein | Coenzyme transport and metabolism | 35 | Circular |
| Atu0684 | groES | co-chaperonin GroES | Carbohydrate transport and metabolism | 2 | Circular |
| Atu0685 | - | hypothetical protein | Coenzyme transport and metabolism | 13 | Circular |

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|---------|------|---|---------------------------------------|----|----------|
| Atu0714 | atpI | ATP synthase%2C subunit I | Energy production and conversion | 6 | Circular |
| Atu0715 | atpA | ATP synthase A chain | Energy production and conversion | 9 | Circular |
| Atu0716 | atpC | ATP synthase epsilon chain | Energy production and conversion | 6 | Circular |
| Atu0717 | atpB | ATP synthase B' chain | Energy production and conversion | 10 | Circular |
| Atu0745 | - | hypothetical protein | Coenzyme transport and metabolism | 15 | Circular |
| Atu0787 | dxs | 1-deoxy-D-xylulose-5-phosphate synthase | Amino acid transport and metabolism | 27 | Circular |
| Atu1094 | cysS | cysteinyI-tRNA synthetase | Lipid transport and metabolism | 23 | Circular |
| Atu1101 | fabD | malonyl-CoA:acyl carrier protein transacylase | Nucleotide transport and metabolism | 15 | Circular |
| Atu1128 | - | hypothetical protein | Lipid transport and metabolism | 24 | Circular |
| Atu1161 | - | aspartyl-tRNA synthetase | Coenzyme transport and metabolism | 24 | Circular |
| Atu1169 | parC | topoisomerase IV subunit A | Coenzyme transport and metabolism | 33 | Circular |
| Atu1171 | hemB | delta-aminolevulinic acid dehydratase | Coenzyme transport and metabolism | 19 | Circular |
| Atu1178 | ribC | riboflavin synthase alpha chain | Lipid transport and metabolism | 10 | Circular |
| Atu1244 | nusB | Putative transcription antitermination protein NusB | Amino acid transport and metabolism | 8 | Circular |
| Atu1283 | rplM | 50S ribosomal protein L13 | Energy production and conversion | 12 | Circular |
| Atu1284 | clpX | ATP-dependent Clp protease%2C ATP-binding subunit | Coenzyme transport and metabolism | 20 | Circular |
| Atu1345 | rne | ribonuclease E | Carbohydrate transport and metabolism | 30 | Circular |
| Atu1352 | - | hypothetical protein | Coenzyme transport and metabolism | 3 | Circular |
| Atu1353 | mrcA | penicillin-binding protein 1a | Coenzyme transport and metabolism | 46 | Circular |
| Atu1354 | prfB | peptide chain release factor 2 | Coenzyme transport and metabolism | 18 | Circular |
| Atu1376 | - | hypothetical protein | Nucleotide transport and metabolism | 13 | Circular |
| Atu1378 | folB | dihydroneopterin aldolase | Lipid transport and metabolism | 9 | Circular |
| Atu1379 | folK | diphosphokinase | Lipid transport and metabolism | 9 | Circular |
| Atu1383 | uppS | undecaprenyl pyrophosphate synthase | Lipid transport and metabolism | 14 | Circular |
| Atu1429 | lpxA | acyltransferase | Energy production and conversion | 9 | Circular |
| Atu1430 | - | hypothetical protein | Energy production and conversion | 16 | Circular |
| Atu1436 | kdsA | 3-deoxy-8-phosphooctulonate synthase | Coenzyme transport and metabolism | 9 | Circular |
| Atu1443 | pdhA | pyruvate dehydrogenase alpha subunit | Lipid transport and metabolism | 21 | Circular |
| Atu1498 | ispF | bifunctional synthase protein | Nucleotide transport and metabolism | 14 | Circular |

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|---------|------|---|--|----|----------|
| Atu1574 | tmk | thymidilate kinase | Energy production and conversion | 8 | Circular |
| Atu1595 | ssb | single-strand DNA binding protein | Energy production and conversion | 12 | Circular |
| Atu1596 | - | hypothetical protein | Lipid transport and metabolism | 6 | Circular |
| Atu1597 | nah | salicylate hydroxylase | Lipid transport and metabolism | 20 | Circular |
| Atu1618 | msbB | lipid A biosynthesis lauroyl acyltransferase | Nucleotide transport and metabolism | 17 | Circular |
| Atu1657 | fabF | 3-oxoacyl-(acyl-carrier-protein) synthase II | Coenzyme transport and metabolism | 17 | Circular |
| Atu1681 | fabF | 3-oxoacyl-(acyl-carrier-protein) synthase II | Coenzyme transport and metabolism | 9 | Circular |
| Atu1710 | serS | seryl-tRNA synthetase | Energy production and conversion | 17 | Circular |
| Atu1749 | - | hypothetical protein | Coenzyme transport and metabolism | 31 | Circular |
| Atu1773 | argS | arginyl-tRNA synthetase | Energy production and conversion | 35 | Circular |
| Atu1821 | glmS | glucosamine-fructose-6-phosphate aminotransferase | Amino acid transport and metabolism | 32 | Circular |
| Atu1870 | sufC | ABC transporter ATP-binding protein | Energy production and conversion | 13 | Circular |
| Atu1926 | rplQ | 50S ribosomal protein L17 | Nucleotide transport and metabolism | 7 | Circular |
| Atu2047 | rplK | 50S Ribosomal Protein L11 | Nucleotide transport and metabolism | 9 | Circular |
| Atu2178 | mraW | S-adenosyl methionine-dependent methyltransferase | Amino acid transport and metabolism | 6 | Circular |
| Atu2218 | rpoD | RNA polymerase sigma factor RpoD | Amino acid transport and metabolism | 22 | Circular |
| Atu2421 | petP | transcriptional regulator%2C MarR family | Carbohydrate transport and metabolism | 8 | Circular |
| Atu2488 | - | hypothetical protein | Inorganic ion transport and metabolism | 16 | Circular |
| Atu2490 | ctrA | two component response regulator | Amino acid transport and metabolism | 13 | Circular |
| Atu2612 | - | tRNA-Ala | Lipid transport and metabolism | 1 | Circular |
| Atu2613 | - | tRNA-Ile | Coenzyme transport and metabolism | 5 | Circular |
| Atu2621 | - | 16S ribosomal RNA | Energy production and conversion | 74 | Circular |
| Atu2622 | chvH | elongation factor P | Energy production and conversion | 10 | Circular |
| Atu2623 | dxr | 1-deoxy-D-xylulose 5-phosphate reductoisomerase | Energy production and conversion | 15 | Circular |
| Atu2624 | hemA | 5-aminolevulinate synthase | Energy production and conversion | 23 | Circular |
| Atu2625 | atpC | ATP synthase epsilon chain | Energy production and conversion | 8 | Circular |
| Atu2652 | atpG | ATP synthase gamma chain | Coenzyme transport and metabolism | 12 | Circular |
| Atu2653 | atpA | ATP synthase alpha chain | Coenzyme transport and metabolism | 31 | Circular |
| Atu2667 | atpH | ATP Synthase delta chain | Amino acid transport and metabolism | 10 | Circular |

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|---------|-------|--|---------------------------------------|----|----------|
| Atu2695 | hemC | porphobilinogen deaminase | Amino acid transport and metabolism | 10 | Circular |
| Atu2698 | argG | argininosuccinate synthase | Amino acid transport and metabolism | 21 | Circular |
| Atu2717 | dapF | diaminopimelate epimerase | Coenzyme transport and metabolism | 9 | Circular |
| Atu2778 | leuS | leucyl-tRNA synthetase | Coenzyme transport and metabolism | 39 | Circular |
| Atu2779 | - | hypothetical protein | Amino acid transport and metabolism | 10 | Circular |
| Atu2780 | - | hypothetical protein | Amino acid transport and metabolism | 16 | Circular |
| Atu2835 | parB1 | chromosome Partitioning Protein | Coenzyme transport and metabolism | 22 | Circular |
| Atu8148 | - | hypothetical protein | Coenzyme transport and metabolism | 16 | Circular |
| Atu3508 | ubiG | 3-demethylubiquinone-9 3-methyltransferase | Coenzyme transport and metabolism | 13 | Linear |
| Atu3599 | argH | argininosuccinate lyase | Amino acid transport and metabolism | 19 | Linear |
| Atu3630 | accA | acetyl-CoA subunit alpha | Lipid transport and metabolism | 17 | Linear |
| Atu3709 | glmM | phosphoglucosamine mutase | Carbohydrate transport and metabolism | 33 | Linear |
| Atu3736 | tktA | transketolase | Carbohydrate transport and metabolism | 19 | Linear |
| Atu3771 | hemH | ferrochelatase | Coenzyme transport and metabolism | 29 | Linear |

POORLY CHARACTERIZED

| | | | | | |
|---------|------|-------------------------------------|----------------------------------|-----|----------|
| Atu0026 | - | hypothetical protein | General function prediction only | 35 | Circular |
| Atu0053 | - | 16S ribosomal RNA | Function unknown | 74 | Circular |
| Atu0054 | - | tRNA-Ile | Function unknown | 5 | Circular |
| Atu0055 | - | tRNA-Ala | Function unknown | 1 | Circular |
| Atu0056 | - | hypothetical protein | Function unknown | 14 | Circular |
| Atu0057 | - | 23S ribosomal RNA | Function unknown | 124 | Circular |
| Atu0058 | - | hypothetical protein | Function unknown | 11 | Circular |
| Atu0059 | - | 5S ribosomal RNA | Function unknown | 3 | Circular |
| Atu0060 | - | tRNA-Met | Function unknown | 2 | Circular |
| Atu0078 | - | hypothetical protein | Function unknown | 11 | Circular |
| Atu0319 | aarF | ubiquinone biosynthesis protein | General function prediction only | 25 | Circular |
| Atu0333 | - | ABC transporter ATP-binding protein | General function prediction only | 15 | Circular |
| Atu0334 | - | hypothetical protein | Function unknown | 11 | Circular |

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|---------|------|--|----------------------------------|----|----------|
| Atu0335 | - | hypothetical protein | Function unknown | 14 | Circular |
| Atu0340 | - | SAM dependent methyltransferase | General function prediction only | 12 | Circular |
| Atu0347 | mviN | virulence factor MviN | General function prediction only | 25 | Circular |
| Atu0383 | cgpA | GTP-binding protein | General function prediction only | 9 | Circular |
| Atu0386 | rpmH | 50S ribosomal protein L34 | Function unknown | 4 | Circular |
| Atu0389 | - | hypothetical protein | Function unknown | 13 | Circular |
| Atu0426 | pstB | ABC transporter protein | Function unknown | 16 | Circular |
| Atu0427 | - | hypothetical protein | Function unknown | 7 | Circular |
| Atu0541 | murA | UDP-N-acetylglucosamine | Function unknown | 16 | Circular |
| Atu0623 | zwf | glucose-6-phosphate 1-dehydrogenase | Function unknown | 20 | Circular |
| Atu0633 | ipk | 4-diphosphocytidyl-2C-methyl-D-erythritol kinase | General function prediction only | 9 | Circular |
| Atu0713 | lpxK | tetraacyldisaccharide 4'-kinase | Function unknown | 13 | Circular |
| Atu1036 | sipf | prokaryotic type I signal peptidase | General function prediction only | 11 | Circular |
| Atu1100 | dnaC | replicative DNA helicase | Function unknown | 33 | Circular |
| Atu1107 | - | hypothetical protein | General function prediction only | 15 | Circular |
| Atu1108 | ostA | organic solvent tolerance protein | General function prediction only | 72 | Circular |
| Atu1231 | ribH | riboflavin synthase beta chain | Function unknown | 8 | Circular |
| Atu1306 | - | ABC transporter | Function unknown | 20 | Circular |
| Atu1385 | omp1 | group 1 outer membrane protein precursor | Function unknown | 70 | Circular |
| Atu1431 | lpxB | lipid A-disaccharide synthase | Function unknown | 23 | Circular |
| Atu1450 | pdhB | pyruvate dehydrogenase beta subunit | General function prediction only | 23 | Circular |
| Atu1573 | holB | DNA polymerase III%2C delta prime subunit | Function unknown | 12 | Circular |
| Atu1810 | folE | GTP cyclohydrolase I | Function unknown | 9 | Circular |
| Atu1811 | - | hypothetical protein | Function unknown | 6 | Circular |
| Atu1826 | nifs | cysteine desulfurase | General function prediction only | 29 | Circular |
| Atu1964 | rplA | 50S ribosomal protein L1 | Function unknown | 10 | Circular |
| Atu2084 | secE | secretion protein | General function prediction only | 5 | Circular |
| Atu2101 | mraY | phospho-N-acetylmuramoyl-pentapeptide- transferase | Function unknown | 15 | Circular |
| Atu2103 | murE | UDP-diaminopimelate ligase | Function unknown | 19 | Circular |

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|---------|------------|--------------------------------------|----------------------------------|-----|----------|
| Atu2109 | pbpB1 | penicillin binding protein 2 | Function unknown | 22 | Circular |
| Atu2430 | petR | two component response regulator | Function unknown | 8 | Circular |
| Atu2433 | prsA | ribose-phosphate pyrophosphokinase | Function unknown | 17 | Circular |
| Atu2491 | - | hypothetical protein | Function unknown | 13 | Circular |
| Atu2539 | - | hypothetical protein | Function unknown | 15 | Circular |
| Atu2540 | cah | carbonate dehydratase | Function unknown | 10 | Circular |
| Atu2541 | asd | aspartate-semialdehyde dehydrogenase | Function unknown | 16 | Circular |
| Atu2542 | - | hypothetical protein | Function unknown | 9 | Circular |
| Atu2543 | - | tRNA-Met | Function unknown | 2 | Circular |
| Atu2544 | - | 5S ribosomal RNA | Function unknown | 3 | Circular |
| Atu2545 | - | hypothetical protein | Function unknown | 11 | Circular |
| Atu2546 | - | 23S ribosomal RNA | Function unknown | 125 | Circular |
| Atu2547 | - | hypothetical protein | Function unknown | 14 | Circular |
| Atu2749 | tyrA | chorismate mutase | Function unknown | 7 | Circular |
| Atu2781 | ftsK1 | putative ftsK cell division protein | General function prediction only | 37 | Circular |
| Atu2834 | rplU | 50S ribosomal protein L21 | Function unknown | 4 | Circular |
| Atu8016 | parA | Chromosome partitioning protein | Function unknown | 25 | Circular |
| Atu8142 | rho | transcription termination factor Rho | Function unknown | 26 | Circular |
| Atu8182 | hemE | uroporphyrinogen decarboxylase | Function unknown | 22 | Circular |
| Atu3001 | - | hypothetical protein | Function unknown | 44 | Linear |
| Atu3221 | - | hypothetical protein | Function unknown | 16 | Linear |
| Atu3712 | - | hypothetical protein | Function unknown | 17 | Linear |
| Atu3761 | - | hypothetical protein | Function unknown | 11 | Linear |
| Atu3824 | - | hypothetical protein | Function unknown | 37 | Linear |
| Atu3922 | repC | replication initiation protein RepC | Function unknown | 28 | Linear |
| Atu8080 | ctRNA_p42d | ctRNA | Function unknown | 2 | Linear |
| Atu3937 | - | 16S ribosomal RNA | Function unknown | 74 | Linear |
| Atu3938 | - | tRNA-Ile | Function unknown | 5 | Linear |
| Atu3939 | - | tRNA-Ala | Function unknown | 1 | Linear |

| | | | | | |
|---------|------|--|----------------------------------|-----|--------|
| Atu3940 | - | hypothetical protein | Function unknown | 14 | Linear |
| Atu3941 | - | 23S ribosomal RNA | Function unknown | 124 | Linear |
| Atu3942 | - | hypothetical protein | Function unknown | 11 | Linear |
| Atu4057 | exoT | succinoglycan biosynthesis transport protein | General function prediction only | 36 | Linear |
| Atu4059 | exoV | succinoglycan biosynthesis protein | Function unknown | 12 | Linear |
| Atu4065 | - | hypothetical protein | Function unknown | 7 | Linear |
| Atu4180 | - | 16S ribosomal RNA | Function unknown | 74 | Linear |
| Atu4181 | - | tRNA-Ile | Function unknown | 5 | Linear |
| Atu4182 | - | tRNA-Ala | Function unknown | 1 | Linear |
| Atu4183 | - | hypothetical protein | Function unknown | 14 | Linear |
| Atu4184 | - | 23S ribosomal RNA | Function unknown | 122 | Linear |
| Atu4185 | - | hypothetical protein | Function unknown | 13 | Linear |
| Atu4350 | - | hypothetical protein | Function unknown | 33 | Linear |
| Atu4351 | - | hypothetical protein | Function unknown | 40 | Linear |
| Atu4852 | - | hypothetical protein | Function unknown | 35 | Linear |

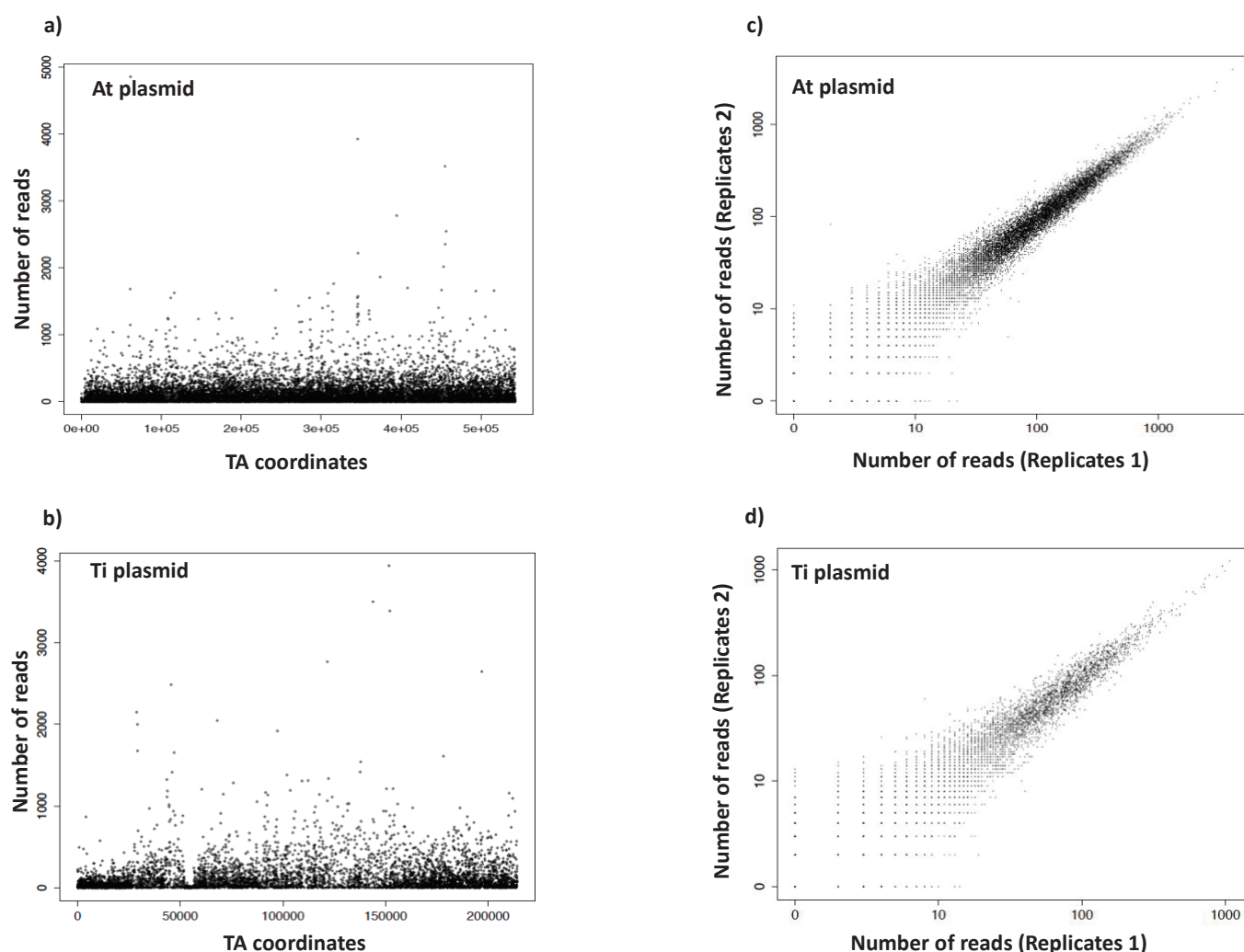


Figure S1. Quality control of the transposon sequencing (Tn-seq) *Agrobacterium fabrum* C58 libraries for the pAT and the pTi plasmids. Frequency and distribution of transposon sequence reads along (a) the pAT and (b) the pTi plasmids. Biological reproducibility of the results for (c) the pAT and (d) the pTi plasmids. Analysis of DNA samples corresponding to two independent cultures of two independent mutant library. The calculated Pearson correlation coefficient were 0,98 for each replicon.

Acknowledgments

We knowledge funding from “La Fédération de Recherche (FR) BioEnviS, Université Lyon 1” and the French National Research Agency (ANR-18-CE32-0005, DIORE). S. Lecomte received doctoral grant from the French Ministère de l’Education nationale de l’Enseignement Supérieur et de la Recherche. Quality controls of the Tn-seq DNA libraries were performed at Biofidal (Vaulx-en-Velin, France), Tn-seq DNA libraries were sequenced at MGX (CNRS Sequencing Service, Montpellier, France), plants were grown at the greenhouse platform (FR BioEnviS, University Lyon 1). We knowledge A. Dubost and D. Abrouk from Ibio platform (UMR 5557, University Lyon 1) for Lasergene Genvision representations.

Authors contributions

FZH, XN and SL design the experiment. SL performed experiments, LM performed bioinformatic analyses, and EG provide advices for transposon library construction. SL and FZH wrote the article and LM EG and XN reviewed and approved the final manuscript.

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Chapter 3

Are primary metabolites exuded by maize roots used as terminal electron acceptors by *Agrobacterium fabrum* C58 anaerobically?

Preamble

The rhizosphere of plants is an important microbial hotspot (Kuzyakov and Razavi, 2019) through the exudations of root metabolites (Haichar et al. 2014). The rhizosphere microbial community is shaped by the **releasing of 16–50% of plant photosynthetically fixed carbon** (Haichar et al., 2016, Guyonnet et al., 2017). In addition, the roots of growing plants modify the soil oxygen partial pressure in their immediate vicinity (Kuzyakov and Razavi, 2019). This variation in oxygen concentration constrains **bacteria to adapt their physiology and to develop flexible metabolic pathways**, such as the use of alternative terminal electron acceptors (TEAs) for anaerobic respiration. In rhizosphere, the most characterize anaerobic respiration is denitrification (Zumft, 1997), while root exudates are available and could serve as TEAs. Nevertheless, no data is available on **the use of plant primary metabolites for anaerobic respiration**.

Among rhizobacteria, *Agrobacterium fabrum* C58 is able to colonize efficiently maize rhizosphere (Chèneby et al., 2004) and perform denitrification (Baek et al., 2005). Recently, we demonstrated that *A. fabrum* C58 *napA* mutant strain, impaired in the reduction of nitrate to nitrite, is able to colonize maize roots in anoxic hydroponic system (Lecomte et al., chapter 1). This result suggests that *A. fabrum* C58 is able to use roots exudates as TEAs. Our hypothesis is *A. fabrum* C58 has developed an efficient strategy to colonize maize rhizosphere based on the **diversification of its anaerobic respiration of root exuded primary metabolites**.

This work aimed to evidence the potential use of primary metabolites exuded by maize plants as TEAs for *A. fabrum* C58 anaerobic respiration. To evidence new TEAs, we **set up a new screening method** based on the determination of an electron donor and an acceptor, and on the guaranty of the absence of oxygen. Then, we **identified primary metabolites exuded by maize plant** in hydroponic system and **tested their use as TEAs** in *A. fabrum* C58.

We determine that **glucose is a good electron donor** for *A. fabrum* C58 and our conditions guaranty the **establishment of anaerobic respiration** when it occurs.

Using GC-MS analysis, primary metabolites exuded by maize plant were identified. A total of **19 sugars, 8 amino acids, 5 organic acids** were evidenced, such as glucose, fructose, L-serine and malic acid. Interestingly, glucose has been found in many plant root exudates suggesting its potential use as electron donor by *A. fabrum* C58 rhizobacteria when anaerobic respiration conditions are fulfilled.

For the first time, we evidenced that rhizobacteria, such as ***A. fabrum* C58, are able to perform anaerobic respiration using TEAs identified as primary metabolites exuded by maize roots**, such as xylose, fumarate, fructose, malic acid, succinic acid, L-serine, mannitol and L-alanine.

Identifying genes involved in these anaerobic respiration pathways by transposon sequencing (Tn-seq) and **exploring the role** of these processes in rhizosphere colonization by competition assays will improve our comprehension of the ecological impact of these respirations.

Results of this work are organized in a manuscript.

Are primary metabolites exuded by maize roots used as terminal electron acceptors by *Agrobacterium fabrum* C58 anaerobically?

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Summary

- Around 16-50% of total photosynthetically fixed carbon are transferred by plants into the rhizosphere through root exudates. Among primary metabolites exuded, a variety could serve as terminal electron acceptors (TEAs) for anaerobic respiration. Nevertheless, no data is available on the use of plant primary metabolites for anaerobic respiration.
- We used *Agrobacterium fabrum* C58, a root associated bacterium, able to colonize efficiently maize roots and to perform denitrification function to explore anaerobic respiration abilities. We set up a screening method in microplates to evidence the use of soluble substrates as TEAs. We identified primary metabolites exuded by maize plant in hydroponic system and tested their use as TEAs by *A. fabrum* C58.
- For the first time, we evidenced that rhizobacteria such as *A. fabrum* C58 is able to perform anaerobic respiration using TEAs identified as primary metabolites exuded by maize roots, such as xylose, fumarate, fructose, malic acid, succinic acid, L-serine, mannitol and L-alanine.
- Identifying genes involved in these anaerobic respiration pathways by transposon sequencing (Tn-seq) and exploring the role of these processes in rhizosphere colonization by competition assays will improve our comprehension of the ecological impact of these respirations.

1 Introduction

The ability to secrete a wide range of compounds into the rhizosphere is one of the most remarkable metabolic features of plant roots, with around 16-50% of total photosynthetically fixed carbon being transferred into the rhizosphere through root exudates (Derrien et al., 2004; Haichar et al., 2016). Through the exudation of variety of primary and secondary metabolites, roots may shape and regulate the soil microbial community in their immediate vicinity. In addition, the roots of growing plants modify the soil oxygen partial pressure (Kuzakov & Razavi, 2019). This variation in oxygen concentration constrains bacteria to adapt their physiology and to develop flexible metabolic pathways, such as the use of other compounds than oxygen as terminal electron acceptors (TEAs) for anaerobic respiration. Indeed, bacterial anaerobic respiration is one of the most flexible and diverse metabolic processes (Hong and Gu, 2009). In the rhizosphere, a variety of root exudates could serve as TEAs such as primary metabolites, however, to our knowledge, no data is available on the use of plant primary metabolites for anaerobic respiration. Indeed, until now, denitrification, the anaerobic respiration of nitrate to N₂, through the reduction of nitrite, NO and N₂O (Zumft, 1997), is the most studied one.

Recently, we demonstrated using *Agrobacterium fabrum* C58 that a *napA* mutant strain, impaired in the reduction of nitrate to nitrite, is able to colonize maize roots in anoxic hydroponic system

(Lecomte et al., chapter 1). This result suggests that *A. fabrum* C58 is able to use roots exudates as TEAs. As this strain is preferentially selected in maize rhizosphere (Chèneby et al., 2004), we can assume that *A. fabrum* C58 has developed an efficient strategy to colonize maize rhizosphere based on the diversification of nutrient acquisition and anaerobic respiration of roots exudates.

The aim of this work was to evidence the potential use of primary metabolites exuded by plants for *A. fabrum* C58 anaerobic respiration. To reach this purpose, firstly, we set up a screening method to determine the potential use of carbon and nitrogen sources as TEAs. Secondly, we identified primary metabolites exuded by maize model using gas chromatography-mass spectrometry (GC-MS) analysis and screened them as potential TEAs. Finally, we validated the carbon and nitrogen source candidates in flasks anaerobically as previously described for nitrate respiration (Lecomte et al., chapter 1). This work proposed a new method to screen soluble compounds that can serve as TEAs. For the first time, we demonstrated that plant primary metabolites can serve as TEAs, such as fumarate and serine.

2 Experimental procedures

2.1 Bacterial strains and growth conditions

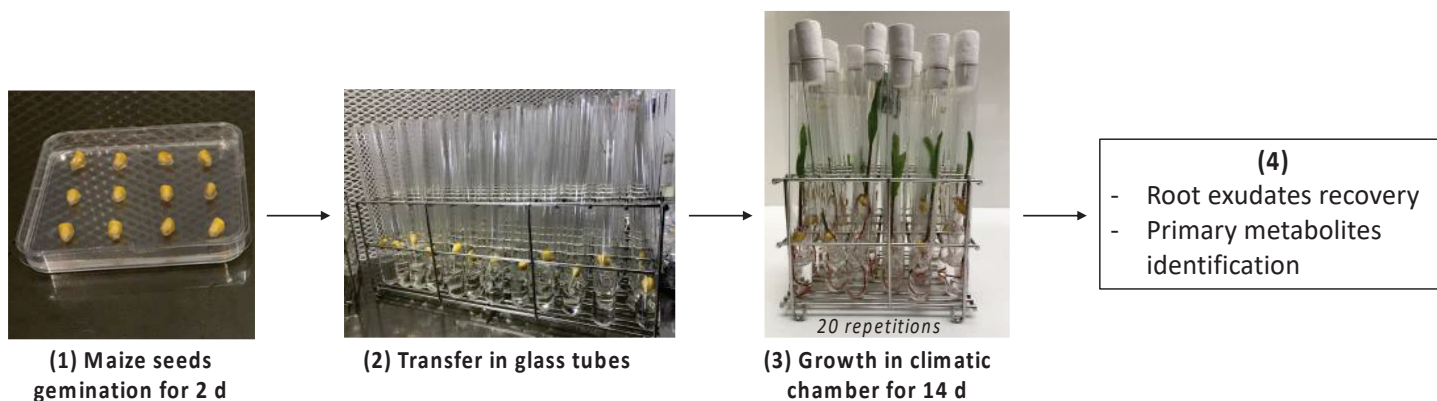
A. fabrum C58 was grown aerobically at 28°C with shaking (180 rpm) on YPG rich medium (per liter: 5 g of yeast extract, 5 g of peptone, 10 g of glucose, pH 7,2). For anaerobic pre-cultures, *A. fabrum* C58 was grown anaerobically at 28°C without shaking on AT minimum medium (80 mM of KH_2PO_4 , 6,5 mM of MgSO_4 , 10 mM of NH_4 , and trace elements (FeSO_4 , CaCl_2 , MnCl_2), supplemented with 20 mM of glucose (electron donor) and 20 mM of nitrate (TEA). Anoxic conditions were attempted by injecting helium in flasks closed hermetically with corks as previously describe in Lecomte et al. (chapter 1).

2.2 Plant growth

Maize seeds were surface-disinfected by immersion for 45 min in 1,2 % commercial bleach, five consecutive bath-washes in sterile-water and finally immersion for 1h30 in sterile water. For pre-germination, seeds were placed on plates containing sterile agar for plants culture at 4 g/L (Sigma-Aldrich, Saint-Quentin Fallavier, France) and incubated in the dark for 48 h at 28°C. Seedlings were then transferred in a sterile glass tube narrow at 5 cm from the bottom and containing 6 mL of sterile plant nutritive solution (0.5 mM of CaSO_4 , 2 mM of NH_4 , 0.5 mM of MgCl_2 , 1 mM of KH_2PO_4 , 0.05 mM of Na_2FETDA , 2.5 mM of MES and trace elements, pH 7) (Fig. 1a). NO_3^- initially present in plant nutritive solution was replaced by NH_4^+ to prevent its presence in collected root exudates. Then, seedlings were grown for 14 days in a climatic chamber with a 16/8h day/night cycle at 24/18°C and 70% hygrometry.

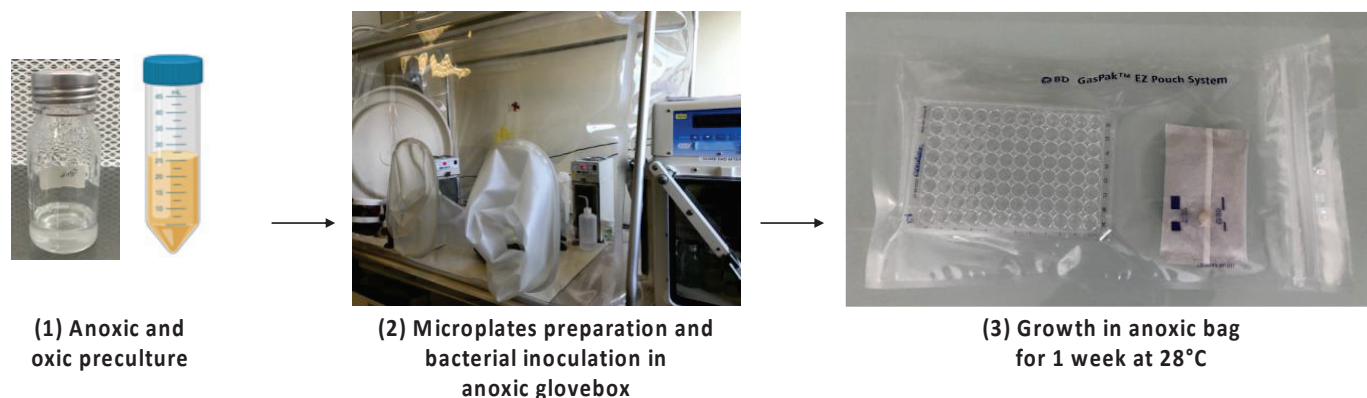
2.3 Collection and identification of maize root exudates

(a)



Experiment performed in 5 replicates

(b)



Experiment performed in 5 replicates

Figure 1. Experimental set up for evidencing new anaerobic respirations. (a) Root exudates recovery and identification. Maize seeds were surface-disinfected and placed on plated for (1) germination for 2 d. Seedlings were (2) transferred in glass tubes containing 6 mL of sterile plant nutritive solution. Then, seedlings were (3) grown for 14 d in a climatic chamber. Finally, roots exudates were (4) collected and identified by GC-MS analysis. Experimentations were performed in 5 replicates. **(b) Set up of growth conditions to evidence new TEAs.** *A. fabrum* C58 strains were grown (1) anaerobically in anoxic flasks for WT and aerobically for C58 Δ *napA*. Microplate manipulations were (2) performed in anoxic glovebox. To prolong anoxic condition outside the glovebox, microplates were (3) incubated in gas pack bags for 1 week at 28°C. Finally, growth was measured by absorbance at OD 600 nm using spectrophotometer (TECAN).

2.3.1 Root exudates recovery

After 14 days of growth, maize plants were removed from the glass tubes and the nutritive solution containing plant exudates were filtered at 0.25 μ m to remove cell debris (Fig. 1a). One replicate corresponds to the filtered nutritive solution from 20 plants. Five replicates were performed. All collected exudates were stored at -80°C before lyophilization (Christ Alpha™ 1-4 freeze dryer, 48 h).

2.3.2 Chemical derivatization and GC-MS analysis

Before derivatization, 1.1 mg of freeze-dried exudates was placed for 30 min in a CentriVap® concentrator (CentriVap Concentrator Labconco®) to remove residual humidity.

1.1 mg of freeze-dried exudates were derivatized for 90 min at 30 °C using 40 µL of O-methylhydroxylamine hydrochloride (MeOx) solution (20 mg/mL MeOx dissolved in pyridine) per sample. This was followed by trimethylsilylation with 70 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) per sample for 30 min at 37 °C.

GC-MS analyses were performed on derivatized samples using an Agilent Technologies 7890A GC system coupled to a 7000A EI-Triple Quad mass spectrometer, using a DB-5MS capillary column (60 m x 0.25 mm x 0.25 µm, Agilent Technologies) with helium as vector gas at a constant flow of 2.3 mL/min. Sample aliquots of 1 µL were injected in splitless mode and with a 40:1 split. The inlet temperature was 290 °C and the oven temperature conditions were as follows: 70 °C for 3 min, ramping up to 300 °C at a rate of 4 °C/min, and holding at 300 °C for 5 min. Total cycle time was 65.5 min. The ionization energy was 70 eV in electron impact mode. The transfer line and ion source temperatures were set at 310 °C and 230 °C, respectively. Mass spectra were obtained with a mass range of 35-500 m/z.

Metabolites were annotated by comparing mass spectra with databases (NIST02/ CNRS/WILEY275 and GOLM) considering a match score > 80. When possible, comparisons with standards were done to confirm metabolite identity.

2.4 Evidence of new anaerobic respirations

2.4.1 Set-up of growth conditions to evidence new TEAs

A. fabrum C58 strains were pre-cultured on AT minimal medium supplemented with glucose and nitrate at 20 mM each. Pre-cultures of C58 WT were grown anaerobically in anoxic flasks, whereas, C58 Δ *napA* were grown aerobically (Fig. 1b).

Microplate manipulations were performed in an anoxic glovebox (Coy laboratory products, Michigan, USA) to ensure anaerobiosis (Fig. 1b). Residual dissolved oxygen was not removed to allow anaerobic respiration induction in *A. fabrum* C58 (Bakken et al., 2012). We used unsupplemented AT minimum medium, AT minimum medium supplemented with 20 mM of glucose, AT minimum medium supplemented with 20 mM of glucose and 20 mM of nitrate. In each well from the microplate, 175 µL of medium were added and 25 µL of either C58 WT or C58 Δ *napA* pre-culture were inoculated at final DO 0,08. Microplates were put in gas pack bags to prolong anoxic condition outside the glovebox. Microplates were incubated for 1 week at 28°C before measuring absorbance at OD 600 nm using spectrophotometer (TECAN). All experiments were performed in 3 replicates.

2.4.2 Screening of C and N substrates used as TEAs

The conditions set up previously were applied to screen the use of different C and N substrates as putative TEAs in *A. fabrum* C58 WT. AT medium supplemented with glucose (20 mM) as electron donor was used in all conditions. The carbon or nitrogen sources (20 mM) tested as potential TEAs were: D-fructose, D-xylose, D-maltose, D-rhamnose, D-cellobiose, gentiobiose, D-mannitol, melibiose, malic

acid, citric acid, fumaric acid, succinic acid, aspartic acid, L-serine, L-alanine and L-leucine. AT minimum media and *A. fabrum* C58 strains used previously for the set up served as controls for this experiment. After 1 week of growth in gas pack bag, absorbance was measured at DO 600 nm (TECAN).

2.5 Statistical analysis

Differences in growth between each TEAs tested, and controls were analyzed using one-way analysis of variance (ANOVA1) followed by a Tukey's HSD test in R studio. Tests were performed in conformity with the assumptions of normality and homogeneity of variance (Shapiro and Bartlett tests were used, respectively). The bacterial growth was figured using "ggplots2" package from R studio.

3 Results and discussion

3.1 A new method to screen C and N sources used as TEAs

The basic anaerobic respiratory process involves the oxidation of a low-redox-potential electron donor, such as carbon substrates, the transfer of electrons through a wide range of cytochromes and the reduction of a high-redox-potential electron acceptor (Lecomte et al., 2018). To screen the potential use of carbon and nitrogen sources as TEAs for anaerobic respirations, we set up a new method based on the determination of an electron donor and an acceptor, and on the guaranty of the absence of oxygen (Fig. 1a). To determine electron donor, AT medium supplemented with glucose was inoculated with *A. fabrum* C58 WT under anoxic condition. No growth was observed (Fig. 2) confirming that glucose is only used as electron donor and could be used for C and N source screening. To ensure the establishment of anaerobic respiration, nitrate, TEA known to be used by *A. fabrum* C58 WT (Baek & Shapleigh, 2005), was supplemented to AT medium with glucose and inoculated with *A. fabrum* C58 WT under anoxic condition. Significant growth was observed compared to AT medium alone inoculated with WT strain (Fig. 2). Supplemental control was performed by growing *A. fabrum* C58 $\Delta napA$ in these conditions to verify anaerobiosis and validate anaerobic respiration establishment. No growth was observed (Fig. 2) as C58 $\Delta napA$ is not able to respire nitrate anaerobically attesting the absence of oxygen. Collectively, these experiments and results ensure the establishment of anaerobic respiration when it occurs.

3.2 Which primary metabolites are exuded by maize?

Metabolite profiling was performed on root exudates of maize plants grown in hydroponic conditions (Fig. 1b). Among detected chromatogram peaks, 32 have been annotated using standards or databases (Fig. 3). Similar profiles were observed for each replicate, demonstrating the good reproducibility of the experiment. A total of 19 sugars, 8 amino acids, 5 organic acids were annotated

(Table 1). Some of these primary metabolites, such as glucose, fructose and sucrose, were already identified in maize root exudates (Carvalhais et al., 2011; Fan et al., 2012). Some of these primary metabolites were also identified in other rhizospheres, such as *Arabidopsis thaliana* (Chaparro et al., 2013), banana (Zhang et al., 2014a) and *Dactylis glomerata* (Guyonnet et al., 2017). For example, amino acids, such as serine, valine, threonine and glycine, were also exuded by *A. thaliana* plant (Chaparro et al., 2013). Interestingly, glucose has been found in many plant root exudates suggesting its potential use as electron donor by *A. fabrum* C58 rhizobacteria when anaerobic respiration conditions are fulfilled.

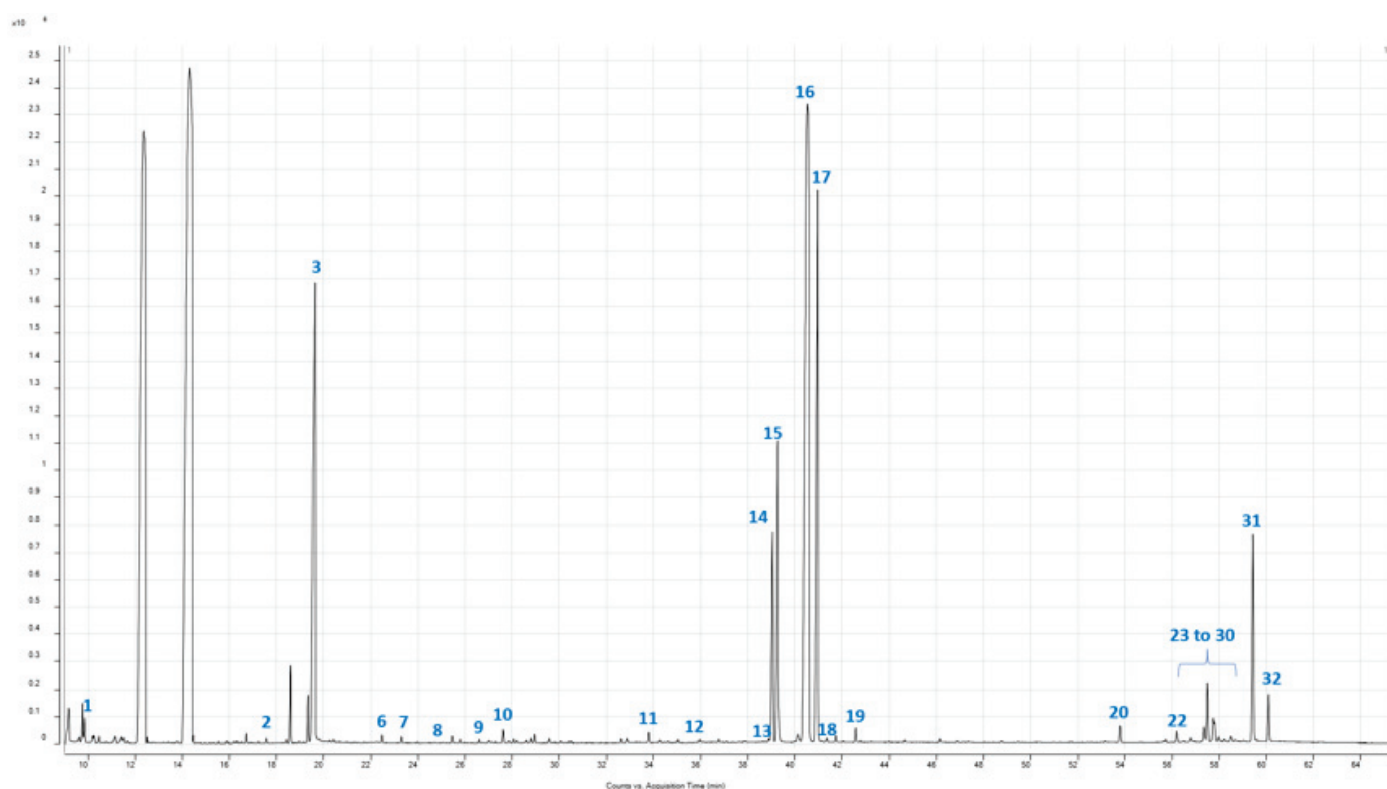


Figure 3. Chromatogram from primary metabolites issued from maize plant grown under hydroponic conditions. Peaks with a number from 1 to 32 corresponded to primary metabolites that were identified. Peaks with star corresponded to derivatization solvents.

Table 1: Identification of primary metabolites retrieved from maize root exudates grown under hydroponic conditions. Primary metabolites were annotated by comparison with databases and/or standards using their retention index (RI) and mass spectrometry.

| Peak | RT (min) | RI | Compound Identification | Class | Formula | Mass (m/z) |
|------|----------|------|------------------------------|------------------|---|------------|
| 1 | 9,862 | nd | nd | Carboxylic acids | C ₂ H ₆ O ₂ | 62 |
| 2 | 17,584 | 1211 | L-Valine | Amino acids | C ₅ H ₁₁ NO ₂ | 117 |
| 3 | 19,654 | 1271 | Phosphoric acid | Inorganic acids | H ₃ O ₄ P | 98 |
| 4 | 20,432 | 1293 | L-Proline | Amino acids | C ₅ H ₉ NO ₂ | 115 |
| 5 | 20,690 | 1300 | L-Glycine | Amino acids | C ₂ H ₅ NO ₂ | 75 |
| 6 | 22,484 | 1354 | L-Serine | Amino acids | C ₃ H ₇ NO ₃ | 105 |
| 7 | 23,306 | 1379 | L-Threonine | Amino acids | C ₄ H ₉ NO ₃ | 119 |
| 8 | 24,695 | 1422 | β-Alanine | Amino acids | C ₃ H ₇ NO ₂ | 89 |
| 9 | 26,590 | 1482 | Malic acid | Carboxylic acids | C ₄ H ₆ O ₅ | 134 |
| 10 | 27,603 | 1515 | 5-Oxoproline | Amino acids | C ₅ H ₇ NO ₃ | 129 |
| 11 | 33,797 | 1730 | Putrescine | Diamine | C ₄ H ₁₂ N ₂ | 88 |
| 12 | 35,955 | 1810 | Citric acid | Carboxylic acids | C ₆ H ₈ O ₇ | 192 |
| 13 | 38,872 | 1924 | Sorbitol | Monosaccharides | C ₆ H ₁₄ O ₆ | 182 |
| 14 | 39,038 | 1931 | Fructose (TMS derivatized 1) | Monosaccharides | C ₆ H ₁₂ O ₆ | 180 |
| 15 | 39,257 | 1940 | Fructose (TMS derivatized 2) | Monosaccharides | C ₆ H ₁₂ O ₆ | 180 |
| 16 | 40,523 | 1992 | Glucose (TMS derivatized 1) | Monosaccharides | C ₆ H ₁₂ O ₆ | 180 |
| 17 | 40,960 | 2010 | Glucose (TMS derivatized 2) | Monosaccharides | C ₆ H ₁₂ O ₆ | 180 |
| 18 | 41,345 | 2027 | Galactaric acid | Carboxylic acids | C ₆ H ₁₀ O ₈ | 210 |
| 19 | 42,568 | 2079 | Myo-inositol | Monosaccharides | C ₆ H ₁₂ O ₆ | 180 |
| 20 | 53,793 | 2621 | Sucrose | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 21 | 55,732 | 2727 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 22 | 56,195 | 2753 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 23 | 56,772 | 2786 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 24 | 57,340 | 2819 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 25 | 57,488 | 2827 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 26 | 57,733 | 2841 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 27 | 57,794 | 2845 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 28 | 57,960 | 2855 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 29 | 58,205 | 2869 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 30 | 58,475 | 2884 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 31 | 59,419 | 2941 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 32 | 60,074 | 2981 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |

To test the use of primary metabolite root exudates as TEAs and hence evidence new anaerobic respirations, we selected among identified primary metabolites (Table 1): 6 sugars (fructose, 4 disaccharides (D-cellobiose, D-gentiobiose, melibiose and maltose), and mannitol, a substrate similar to sorbitol), 2 organic acids (malic acid and citric acid) and 2 amino acids (L-serine and L-alanine).

3.3 *A. fabrum* C58 is able to use some plant primary metabolites as TEAs

To evidence the use of certain C and N sources as TEAs by *A. fabrum* C58, we applied the screening method set up previously. Sixteen C and N sources were chosen: 10 substrates from identified maize primary metabolite root exudates (Table 1) and 6 substrates (xylose, rhamnose, fumarate, succinate, leucine, aspartate) not identified in our study but known to be exuded by maize plant (Fan et al., 2012).

Controls ensuring anaerobiosis and anaerobic respiration establishment previously set up were performed. To evidence the use of substrates as TEAs, we compared growth on glucose and on each tested substrate. As seen previously, no growth was observed on glucose as TEAs (Fig. 4). Relative to the glucose control, significant growth was observed on 3 sugars, 3 carboxylic acids and 3 amino acids (Fig. 4). In descending order, the magnitude of *A. fabrum* C58 growth was as follows: xylose/fumaric acid > fructose/malic acid/succinic acid/L-serine > mannitol >> L-alanine. No growth was observed on maltose, rhamnose, D-cellobiose, gentiobiose, melibiose, citric acid, aspartate and leucine (Fig. 4). The growth of *A. fabrum* C58 on xylose, fructose, mannitol, fumaric acid, malic acid, succinic acid, L-serine and L-alanine suggests their use as TEAs for anaerobic respiration.

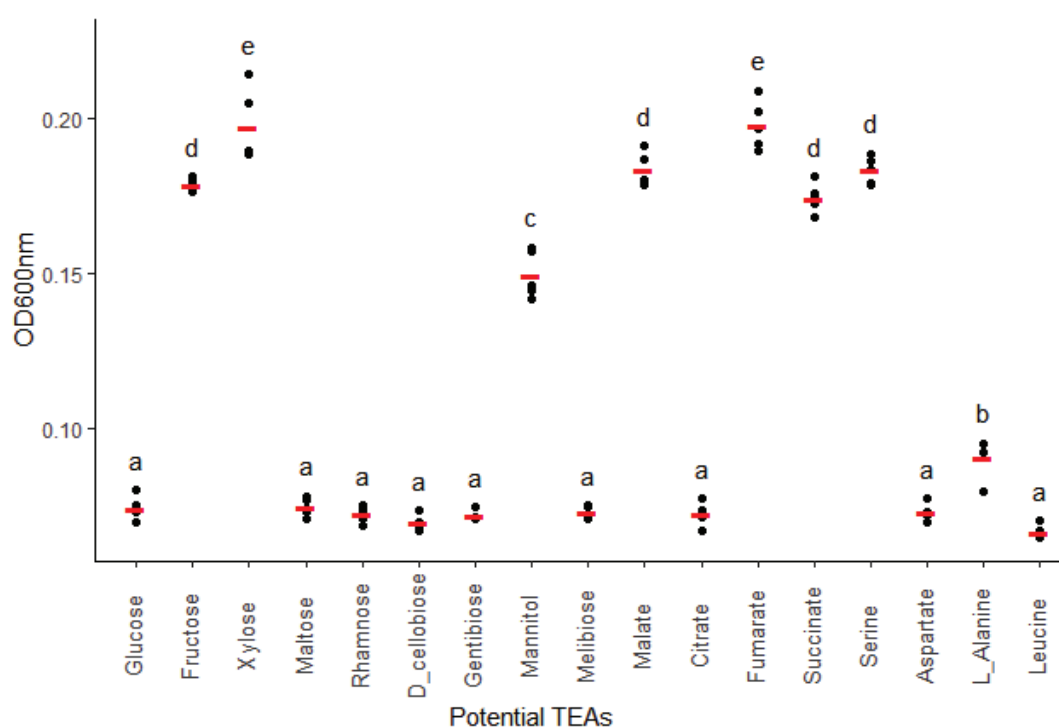


Figure 1. Growth of *A. fabrum* C58 on carbon and nitrogen sources as TEAs in microplates under anoxic conditions. Microplate manipulations were performed in anoxic glovebox. AT minimum medium supplemented with glucose and AT minimum medium supplemented with glucose and a potential TEA (xylose, fructose, maltose, rhamnose, D-cellobiose, gentiobiose, melibiose, mannitol, malic acid, fumaric acid, succinic acid, citric acid, aspartic acid, L-serine, L-alanine, and leucine) were inoculated with *A. fabrum* C58 WT. Growth of this strain was measured after 1 week at 28°C under anoxic conditions using spectrophotometer (TECAN). All experiments were performed in 5 replicates. Significant difference with AT glucose is figured by letters.

Interestingly, fumaric acid exuded by various plant species such as maize, banana, barley and *D. glomerata* (Jousset et al., 2014; Zhang et al., 2014b; Guyonnet et al., 2017) is used by *A. fabrum* C58 for anaerobic respiration. Fumaric acid respiration has been described only in a few bacteria, such as *E. coli*, *Wolinella succinogenes* and *Shewanella* (Ackrell, 1992; Kröger et al., 2002; Arkhipova & Akimenko, 2005). Fumarate is reduced to succinate, the reverse reaction of succinate to fumarate that occurs aerobically in the tricarboxylic acid cycle (TCA cycle). We evidenced, for the first time, that fumaric acid respiration may occur in *A. fabrum* C58 rhizobacteria and probably in plant rhizosphere. The anaerobic respiration of fumarate in succinate with glucose as electron donor produce enough energy to sustain growth ($\Delta G = -240$ kcal / mol of glucose): fumarate + 2 glucose \rightarrow 2 CO₂ + succinate.

Sugars, such as fructose and xylose, are also used as TEAs by *A. fabrum* C58 rhizobacteria. These anaerobic respirations are not due to fermentative process as *A. fabrum* C58 lacks fermentative genes (Lassalle et al., 2017). Xylose is the second most abundant sugar present in plant lignocellulose after glucose and is frequently found in root exudates (Jones et al., 2004; Vranova et al., 2013). Fructose is also often found in root exudates of plants, such as *Fagus sylvatica* (Zang et al., 2014), potato (Hoysted et al., 2018) and *A. thaliana* (Carvalhais et al., 2015).

A. fabrum C58 is able to colonize efficiently maize rhizosphere (Chèneby et al., 2004). Remarkably, mobility tests demonstrated a highly sensitive chemotaxis system towards sugars exuded by maize in *A. tumefaciens* C58C1 (Loake et al., 1988). Among highly chemoattractant sugars, sucrose, glucose and fructose are recognized by this strain. Collectively, these results suggest that *A. fabrum* C58 could have adapted its chemotaxis system to improve its response to plant root exuded sugars to efficiently colonize rhizosphere. Diversifying the use of carbon-containing and nitrogen-containing exudates through anaerobic respiration, could be considered as a rhizospheric trait allowing competitive advantage as shown for denitrification (Philippot et al., 1995).

In the presence of multiple TEAs in a complex environment, such as the rhizosphere, microorganisms will use TEAs in specific order (Achtnich et al., 1995). Even if this order is specific to each microorganism, they generally prefer to use the more energetic option first (Achtnich et al., 1995). The most energetic anaerobic respiration for rhizobacteria is denitrification (Lecomte et al., 2018, Table 2). However, soil nitrate spatial and temporal availability is highly heterogeneous (Arth & Frenzel, 2000), and plants compete for nitrate uptake with nitrate-reducing bacteria (Moreau et al.,

2015). In nitrate limiting environment, rhizobacteria may adapt and diversify their anaerobic respiration for growth or cell maintenance by using alternative TEAs, such as primary metabolite root exudates.

It is well-known that the free energy released during the electron transfer process in denitrification is more favorable for bacterial growth than that in fumaric acid respiration (Thauer et al., 1989; Yoon et al., 2013). Exploring enzyme kinetics of each identified TEA will determine the energy produced during these anaerobic respirations and hence give clues on anaerobic respiration role in growth and/or cells maintenance.

For the first time, this study highlights that rhizobacteria such as *A. fabrum* C58 is able to perform anaerobic respiration using TEAs identified as primary metabolites exuded by maize roots. Better understanding of these anaerobic respiration pathways is needed, especially mechanisms and genes encoding these processes and their ecological role in rhizosphere. One way to evidence these genes is mutagenesis combined with high sequencing provided by transposon sequencing (Tn-seq) (Royet et al., 2018; Gonzalez-Mula et al., 2019). Growing Tn-seq library, already constructed (Lecomte et al., Chapter 2), on individual identified TEA in anaerobic flasks will reveal which genes are crucial to respire anaerobically this substrate. Performing growth competition assays between mutant of these genes and WT strain will improve our comprehension of ecological role of these anaerobic respirations in rhizosphere.

Acknowledgments

We knowledge funding from “La Fédération de Recherche (FR) BioEnviS, Université Lyon 1” and the French National Research Agency (ANR-18-CE32-0005, DIORE). S. Lecomte received doctoral grant from the French Ministère de l’Education nationale de l’Enseignement Supérieur et de la Recherche. Plants were grown at the greenhouse platform (FR BioEnviS, University Lyon 1). GC-MS analysis was performed at the CESN platform (UMR 5557, University Lyon 1). We knowledge D. Abrouk from Ibio platform (UMR 5557, University Lyon 1) and J. Trumello (UMR7265, CEA Cadarache) for their kind help for statistical analysis.

Authors contributions

SL, XN and FZH set up the experiment. SL performed experiments, AM performed set up experiments. GM performed GC-MS analysis and metabolite annotation. PO provide advices for anaerobic experiment in glovebox. SL and FZH wrote the paper and GM, PO and XN reviewed and approved the final manuscript.

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Chapter 3

Are primary metabolites exuded by maize roots used as terminal electron acceptors by *Agrobacterium fabrum* C58 anaerobically?

Preamble

The rhizosphere of plants is an important microbial hotspot (Kuzyakov and Razavi, 2019) through the exudations of root metabolites (Haichar et al. 2014). The rhizosphere microbial community is shaped by the **releasing of 16–50% of plant photosynthetically fixed carbon** (Haichar et al., 2016, Guyonnet et al., 2017). In addition, the roots of growing plants modify the soil oxygen partial pressure in their immediate vicinity (Kuzyakov and Razavi, 2019). This variation in oxygen concentration constrains **bacteria to adapt their physiology and to develop flexible metabolic pathways**, such as the use of alternative terminal electron acceptors (TEAs) for anaerobic respiration. In rhizosphere, the most characterize anaerobic respiration is denitrification (Zumft, 1997), while root exudates are available and could serve as TEAs. Nevertheless, no data is available on **the use of plant primary metabolites for anaerobic respiration**.

Among rhizobacteria, *Agrobacterium fabrum* C58 is able to colonize efficiently maize rhizosphere (Chèneby et al., 2004) and perform denitrification (Baek et al., 2005). Recently, we demonstrated that *A. fabrum* C58 *napA* mutant strain, impaired in the reduction of nitrate to nitrite, is able to colonize maize roots in anoxic hydroponic system (Lecomte et al., chapter 1). This result suggests that *A. fabrum* C58 is able to use roots exudates as TEAs. Our hypothesis is *A. fabrum* C58 has developed an efficient strategy to colonize maize rhizosphere based on the **diversification of its anaerobic respiration of root exuded primary metabolites**.

This work aimed to evidence the potential use of primary metabolites exuded by maize plants as TEAs for *A. fabrum* C58 anaerobic respiration. To evidence new TEAs, we **set up a new screening method** based on the determination of an electron donor and an acceptor, and on the guaranty of the absence of oxygen. Then, we **identified primary metabolites exuded by maize plant** in hydroponic system and **tested their use as TEAs** in *A. fabrum* C58.

We determine that **glucose is a good electron donor** for *A. fabrum* C58 and our conditions guaranty the **establishment of anaerobic respiration** when it occurs.

Using GC-MS analysis, primary metabolites exuded by maize plant were identified. A total of **19 sugars, 8 amino acids, 5 organic acids** were evidenced, such as glucose, fructose, L-serine and malic acid. Interestingly, glucose has been found in many plant root exudates suggesting its potential use as electron donor by *A. fabrum* C58 rhizobacteria when anaerobic respiration conditions are fulfilled.

For the first time, we evidenced that rhizobacteria, such as ***A. fabrum* C58, are able to perform anaerobic respiration using TEAs identified as primary metabolites exuded by maize roots**, such as xylose, fumarate, fructose, malic acid, succinic acid, L-serine, mannitol and L-alanine.

Identifying genes involved in these anaerobic respiration pathways by transposon sequencing (Tn-seq) and **exploring the role** of these processes in rhizosphere colonization by competition assays will improve our comprehension of the ecological impact of these respirations.

Results of this work are organized in a manuscript.

**Are primary metabolites exuded by maize roots used as terminal electron acceptors
by *Agrobacterium fabrum* C58 anaerobically?**

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Summary

- Around 16-50% of total photosynthetically fixed carbon are transferred by plants into the rhizosphere through root exudates. Among primary metabolites exuded, a variety could serve as terminal electron acceptors (TEAs) for anaerobic respiration. Nevertheless, no data is available on the use of plant primary metabolites for anaerobic respiration.
- We used *Agrobacterium fabrum* C58, a root associated bacterium, able to colonize efficiently maize roots and to perform denitrification function to explore anaerobic respiration abilities. We set up a screening method in microplates to evidence the use of soluble substrates as TEAs. We identified primary metabolites exuded by maize plant in hydroponic system and tested their use as TEAs by *A. fabrum* C58.
- For the first time, we evidenced that rhizobacteria such as *A. fabrum* C58 is able to perform anaerobic respiration using TEAs identified as primary metabolites exuded by maize roots, such as xylose, fumarate, fructose, malic acid, succinic acid, L-serine, mannitol and L-alanine.
- Identifying genes involved in these anaerobic respiration pathways by transposon sequencing (Tn-seq) and exploring the role of these processes in rhizosphere colonization by competition assays will improve our comprehension of the ecological impact of these respirations.

1 Introduction

The ability to secrete a wide range of compounds into the rhizosphere is one of the most remarkable metabolic features of plant roots, with around 16-50% of total photosynthetically fixed carbon being transferred into the rhizosphere through root exudates (Derrien *et al.*, 2004; Haichar *et al.*, 2016). Through the exudation of variety of primary and secondary metabolites, roots may shape and regulate the soil microbial community in their immediate vicinity. In addition, the roots of growing plants modify the soil oxygen partial pressure (Kuzakov & Razavi, 2019). This variation in oxygen concentration constrains bacteria to adapt their physiology and to develop flexible metabolic pathways, such as the use of other compounds than oxygen as terminal electron acceptors (TEAs) for anaerobic respiration. Indeed, bacterial anaerobic respiration is one of the most flexible and diverse metabolic processes (Hong and Gu, 2009). In the rhizosphere, a variety of root exudates could serve as TEAs such as primary metabolites, however, to our knowledge, no data is available on the use of plant primary metabolites for anaerobic respiration. Indeed, until now, denitrification, the anaerobic respiration of nitrate to N₂, through the reduction of nitrite, NO and N₂O (Zumft, 1997), is the most studied one.

Recently, we demonstrated using *Agrobacterium fabrum* C58 that a *napA* mutant strain, impaired in the reduction of nitrate to nitrite, is able to colonize maize roots in anoxic hydroponic system

(Lecomte et al., chapter 1). This result suggests that *A. fabrum* C58 is able to use roots exudates as TEAs. As this strain is preferentially selected in maize rhizosphere (Chèneby et al., 2004), we can assume that *A. fabrum* C58 has developed an efficient strategy to colonize maize rhizosphere based on the diversification of nutrient acquisition and anaerobic respiration of roots exudates.

The aim of this work was to evidence the potential use of primary metabolites exuded by plants for *A. fabrum* C58 anaerobic respiration. To reach this purpose, firstly, we set up a screening method to determine the potential use of carbon and nitrogen sources as TEAs. Secondly, we identified primary metabolites exuded by maize model using gas chromatography-mass spectrometry (GC-MS) analysis and screened them as potential TEAs. Finally, we validated the carbon and nitrogen source candidates in flasks anaerobically as previously described for nitrate respiration (Lecomte et al., chapter 1). This work proposed a new method to screen soluble compounds that can serve as TEAs. For the first time, we demonstrated that plant primary metabolites can serve as TEAs, such as fumarate and serine.

2 Experimental procedures

2.1 Bacterial strains and growth conditions

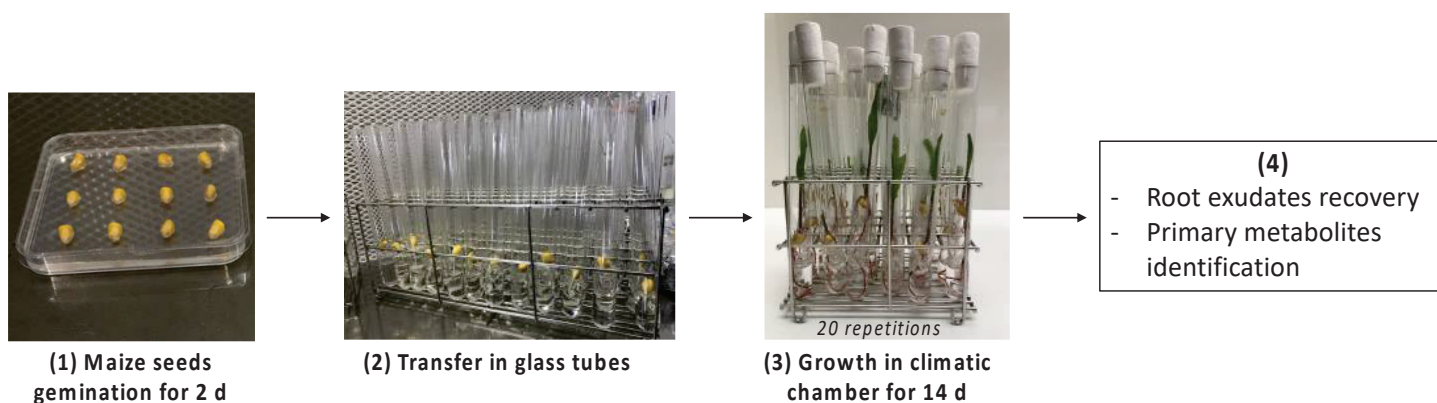
A. fabrum C58 was grown aerobically at 28°C with shaking (180 rpm) on YPG rich medium (per liter: 5 g of yeast extract, 5 g of peptone, 10 g of glucose, pH 7,2). For anaerobic pre-cultures, *A. fabrum* C58 was grown anaerobically at 28°C without shaking on AT minimum medium (80 mM of KH_2PO_4 , 6,5 mM of MgSO_4 , 10 mM of NH_4 , and trace elements (FeSO_4 , CaCl_2 , MnCl_2), supplemented with 20 mM of glucose (electron donor) and 20 mM of nitrate (TEA). Anoxic conditions were attempted by injecting helium in flasks closed hermetically with corks as previously describe in Lecomte et al. (chapter 1).

2.2 Plant growth

Maize seeds were surface-disinfected by immersion for 45 min in 1,2 % commercial bleach, five consecutive bath-washes in sterile-water and finally immersion for 1h30 in sterile water. For pre-germination, seeds were placed on plates containing sterile agar for plants culture at 4 g/L (Sigma-Aldrich, Saint-Quentin Fallavier, France) and incubated in the dark for 48 h at 28°C. Seedlings were then transferred in a sterile glass tube narrow at 5 cm from the bottom and containing 6 mL of sterile plant nutritive solution (0.5 mM of CaSO_4 , 2 mM of NH_4 , 0.5 mM of MgCl_2 , 1 mM of KH_2PO_4 , 0.05 mM of Na_2FETDA , 2.5 mM of MES and trace elements, pH 7) (Fig. 1a). NO_3^- initially present in plant nutritive solution was replaced by NH_4^+ to prevent its presence in collected root exudates. Then, seedlings were grown for 14 days in a climatic chamber with a 16/8h day/night cycle at 24/18°C and 70% hygrometry.

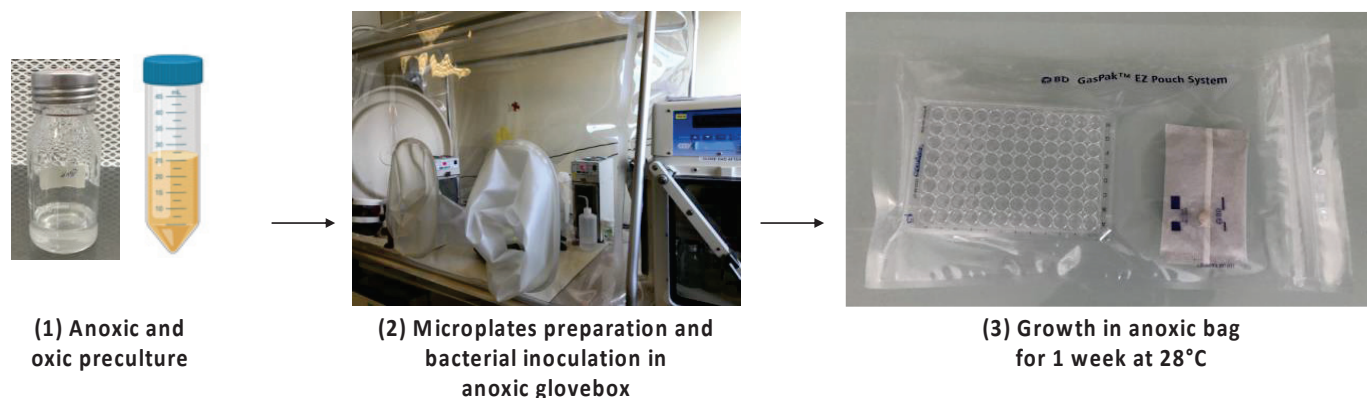
2.3 Collection and identification of maize root exudates

(a)



Experiment performed in 5 replicates

(b)



Experiment performed in 5 replicates

Figure 1. Experimental set up for evidencing new anaerobic respirations. (a) Root exudates recovery and identification. Maize seeds were surface-disinfected and placed on plated for (1) germination for 2 d. Seedlings were (2) transferred in glass tubes containing 6 mL of sterile plant nutritive solution. Then, seedlings were (3) grown for 14 d in a climatic chamber. Finally, roots exudates were (4) collected and identified by GC-MS analysis. Experimentations were performed in 5 replicates. **(b) Set up of growth conditions to evidence new TEAs.** *A. fabrum* C58 strains were grown (1) anaerobically in anoxic flasks for WT and aerobically for C58 $\Delta napA$. Microplate manipulations were (2) performed in anoxic glovebox. To prolong anoxic condition outside the glovebox, microplates were (3) incubated in gas pack bags for 1 week at 28°C. Finally, growth was measured by absorbance at OD 600 nm using spectrophotometer (TECAN).

2.3.1 Root exudates recovery

After 14 days of growth, maize plants were removed from the glass tubes and the nutritive solution containing plant exudates were filtered at 0.25 μm to remove cell debris (Fig. 1a). One replicate corresponds to the filtered nutritive solution from 20 plants. Five replicates were performed. All collected exudates were stored at -80°C before lyophilization (Christ Alpha™ 1-4 freeze dryer, 48 h).

2.3.2 Chemical derivatization and GC-MS analysis

Before derivatization, 1.1 mg of freeze-dried exudates was placed for 30 min in a CentriVap® concentrator (CentriVap Concentrator Labconco®) to remove residual humidity.

1.1 mg of freeze-dried exudates were derivatized for 90 min at 30 °C using 40 µL of O-methylhydroxylamine hydrochloride (MeOx) solution (20 mg/mL MeOx dissolved in pyridine) per sample. This was followed by trimethylsilylation with 70 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) per sample for 30 min at 37 °C.

GC-MS analyses were performed on derivatized samples using an Agilent Technologies 7890A GC system coupled to a 7000A EI-Triple Quad mass spectrometer, using a DB-5MS capillary column (60 m x 0.25 mm x 0.25 µm, Agilent Technologies) with helium as vector gas at a constant flow of 2.3 mL/min. Sample aliquots of 1 µL were injected in splitless mode and with a 40:1 split. The inlet temperature was 290 °C and the oven temperature conditions were as follows: 70 °C for 3 min, ramping up to 300 °C at a rate of 4 °C/min, and holding at 300 °C for 5 min. Total cycle time was 65.5 min. The ionization energy was 70 eV in electron impact mode. The transfer line and ion source temperatures were set at 310 °C and 230 °C, respectively. Mass spectra were obtained with a mass range of 35-500 m/z.

Metabolites were annotated by comparing mass spectra with databases (NIST02/ CNRS/WILEY275 and GOLM) considering a match score > 80. When possible, comparisons with standards were done to confirm metabolite identity.

2.4 Evidence of new anaerobic respirations

2.4.1 Set-up of growth conditions to evidence new TEAs

A. fabrum C58 strains were pre-cultured on AT minimal medium supplemented with glucose and nitrate at 20 mM each. Pre-cultures of C58 WT were grown anaerobically in anoxic flasks, whereas, C58 Δ *napA* were grown aerobically (Fig. 1b).

Microplate manipulations were performed in an anoxic glovebox (Coy laboratory products, Michigan, USA) to ensure anaerobiosis (Fig. 1b). Residual dissolved oxygen was not removed to allow anaerobic respiration induction in *A. fabrum* C58 (Bakken et al., 2012). We used unsupplemented AT minimum medium, AT minimum medium supplemented with 20 mM of glucose, AT minimum medium supplemented with 20 mM of glucose and 20 mM of nitrate. In each well from the microplate, 175 µL of medium were added and 25 µL of either C58 WT or C58 Δ *napA* pre-culture were inoculated at final DO 0,08. Microplates were put in gas pack bags to prolong anoxic condition outside the glovebox. Microplates were incubated for 1 week at 28°C before measuring absorbance at OD 600 nm using spectrophotometer (TECAN). All experiments were performed in 3 replicates.

2.4.2 Screening of C and N substrates used as TEAs

The conditions set up previously were applied to screen the use of different C and N substrates as putative TEAs in *A. fabrum* C58 WT. AT medium supplemented with glucose (20 mM) as electron donor was used in all conditions. The carbon or nitrogen sources (20 mM) tested as potential TEAs were: D-fructose, D-xylose, D-maltose, D-rhamnose, D-cellobiose, gentiobiose, D-mannitol, melibiose, malic

acid, citric acid, fumaric acid, succinic acid, aspartic acid, L-serine, L-alanine and L-leucine. AT minimum media and *A. fabrum* C58 strains used previously for the set up served as controls for this experiment. After 1 week of growth in gas pack bag, absorbance was measured at DO 600 nm (TECAN).

2.5 Statistical analysis

Differences in growth between each TEAs tested, and controls were analyzed using one-way analysis of variance (ANOVA1) followed by a Tukey's HSD test in R studio. Tests were performed in conformity with the assumptions of normality and homogeneity of variance (Shapiro and Bartlett tests were used, respectively). The bacterial growth was figured using "ggplots2" package from R studio.

3 Results and discussion

3.1 A new method to screen C and N sources used as TEAs

The basic anaerobic respiratory process involves the oxidation of a low-redox-potential electron donor, such as carbon substrates, the transfer of electrons through a wide range of cytochromes and the reduction of a high-redox-potential electron acceptor (Lecomte et al., 2018). To screen the potential use of carbon and nitrogen sources as TEAs for anaerobic respirations, we set up a new method based on the determination of an electron donor and an acceptor, and on the guaranty of the absence of oxygen (Fig. 1a). To determine electron donor, AT medium supplemented with glucose was inoculated with *A. fabrum* C58 WT under anoxic condition. No growth was observed (Fig. 2) confirming that glucose is only used as electron donor and could be used for C and N source screening. To ensure the establishment of anaerobic respiration, nitrate, TEA known to be used by *A. fabrum* C58 WT (Baek & Shapleigh, 2005), was supplemented to AT medium with glucose and inoculated with *A. fabrum* C58 WT under anoxic condition. Significant growth was observed compared to AT medium alone inoculated with WT strain (Fig. 2). Supplemental control was performed by growing *A. fabrum* C58 $\Delta napA$ in these conditions to verify anaerobiosis and validate anaerobic respiration establishment. No growth was observed (Fig. 2) as C58 $\Delta napA$ is not able to respire nitrate anaerobically attesting the absence of oxygen. Collectively, these experiments and results ensure the establishment of anaerobic respiration when it occurs.

3.2 Which primary metabolites are exuded by maize?

Metabolite profiling was performed on root exudates of maize plants grown in hydroponic conditions (Fig. 1b). Among detected chromatogram peaks, 32 have been annotated using standards or databases (Fig. 3). Similar profiles were observed for each replicate, demonstrating the good reproducibility of the experiment. A total of 19 sugars, 8 amino acids, 5 organic acids were annotated

(Table 1). Some of these primary metabolites, such as glucose, fructose and sucrose, were already identified in maize root exudates (Carvalhais et al., 2011; Fan et al., 2012). Some of these primary metabolites were also identified in other rhizospheres, such as *Arabidopsis thaliana* (Chaparro et al., 2013), banana (Zhang et al., 2014a) and *Dactylis glomerata* (Guyonnet et al., 2017). For example, amino acids, such as serine, valine, threonine and glycine, were also exuded by *A. thaliana* plant (Chaparro et al., 2013). Interestingly, glucose has been found in many plant root exudates suggesting its potential use as electron donor by *A. fabrum* C58 rhizobacteria when anaerobic respiration conditions are fulfilled.

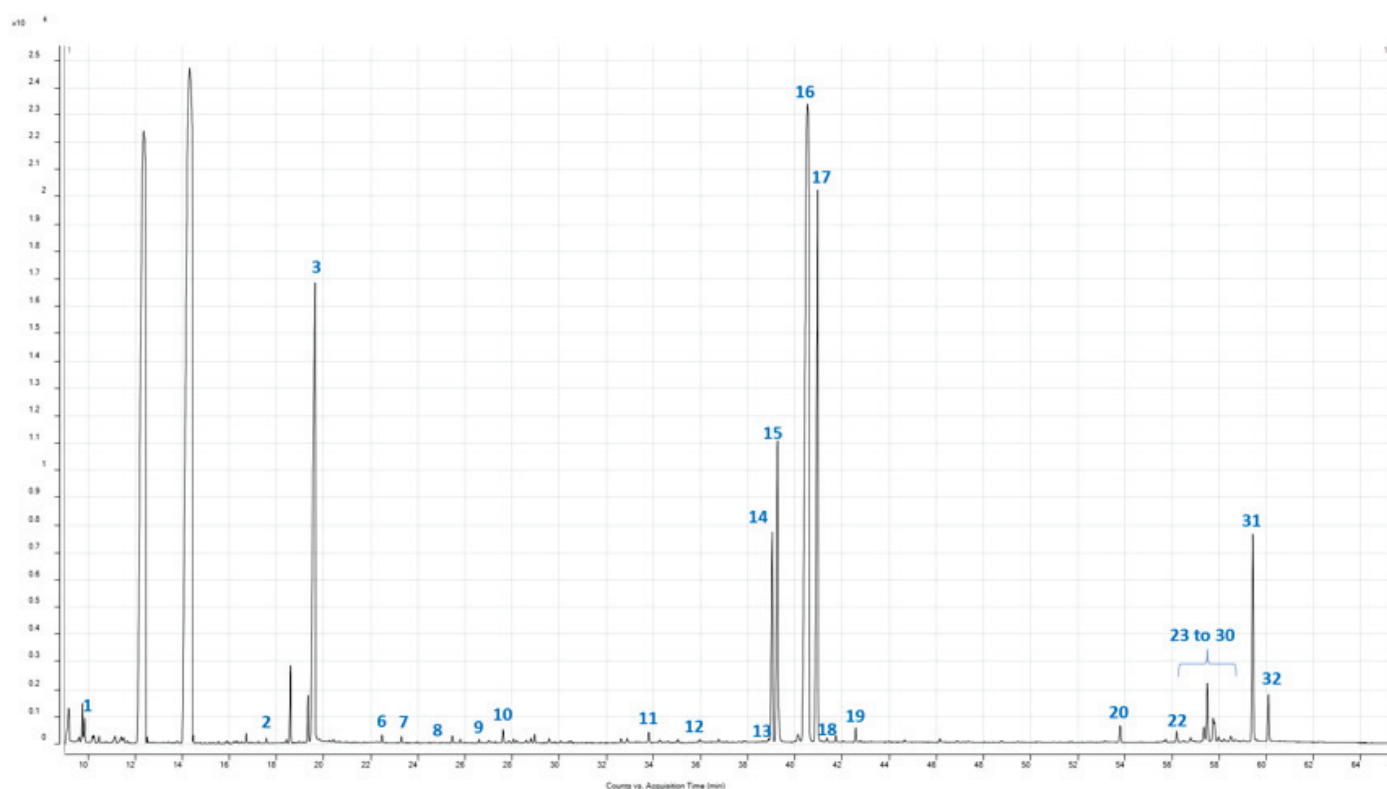


Figure 3. Chromatogram from primary metabolites issued from maize plant grown under hydroponic conditions. Peaks with a number from 1 to 32 corresponded to primary metabolites that were identified. Peaks with star corresponded to derivatization solvents.

Table 1: Identification of primary metabolites retrieved from maize root exudates grown under hydroponic conditions. Primary metabolites were annotated by comparison with databases and/or standards using their retention index (RI) and mass spectrometry.

| Peak | RT (min) | RI | Compound Identification | Class | Formula | Mass (m/z) |
|------|----------|------|------------------------------|------------------|---|------------|
| 1 | 9,862 | nd | nd | Carboxylic acids | C ₂ H ₆ O ₂ | 62 |
| 2 | 17,584 | 1211 | L-Valine | Amino acids | C ₅ H ₁₁ NO ₂ | 117 |
| 3 | 19,654 | 1271 | Phosphoric acid | Inorganic acids | H ₃ O ₄ P | 98 |
| 4 | 20,432 | 1293 | L-Proline | Amino acids | C ₅ H ₉ NO ₂ | 115 |
| 5 | 20,690 | 1300 | L-Glycine | Amino acids | C ₂ H ₅ NO ₂ | 75 |
| 6 | 22,484 | 1354 | L-Serine | Amino acids | C ₃ H ₇ NO ₃ | 105 |
| 7 | 23,306 | 1379 | L-Threonine | Amino acids | C ₄ H ₉ NO ₃ | 119 |
| 8 | 24,695 | 1422 | β-Alanine | Amino acids | C ₃ H ₇ NO ₂ | 89 |
| 9 | 26,590 | 1482 | Malic acid | Carboxylic acids | C ₄ H ₆ O ₅ | 134 |
| 10 | 27,603 | 1515 | 5-Oxoproline | Amino acids | C ₅ H ₇ NO ₃ | 129 |
| 11 | 33,797 | 1730 | Putrescine | Diamine | C ₄ H ₁₂ N ₂ | 88 |
| 12 | 35,955 | 1810 | Citric acid | Carboxylic acids | C ₆ H ₈ O ₇ | 192 |
| 13 | 38,872 | 1924 | Sorbitol | Monosaccharides | C ₆ H ₁₄ O ₆ | 182 |
| 14 | 39,038 | 1931 | Fructose (TMS derivatized 1) | Monosaccharides | C ₆ H ₁₂ O ₆ | 180 |
| 15 | 39,257 | 1940 | Fructose (TMS derivatized 2) | Monosaccharides | C ₆ H ₁₂ O ₆ | 180 |
| 16 | 40,523 | 1992 | Glucose (TMS derivatized 1) | Monosaccharides | C ₆ H ₁₂ O ₆ | 180 |
| 17 | 40,960 | 2010 | Glucose (TMS derivatized 2) | Monosaccharides | C ₆ H ₁₂ O ₆ | 180 |
| 18 | 41,345 | 2027 | Galactaric acid | Carboxylic acids | C ₆ H ₁₀ O ₈ | 210 |
| 19 | 42,568 | 2079 | Myo-inositol | Monosaccharides | C ₆ H ₁₂ O ₆ | 180 |
| 20 | 53,793 | 2621 | Sucrose | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 21 | 55,732 | 2727 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 22 | 56,195 | 2753 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 23 | 56,772 | 2786 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 24 | 57,340 | 2819 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 25 | 57,488 | 2827 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 26 | 57,733 | 2841 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 27 | 57,794 | 2845 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 28 | 57,960 | 2855 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 29 | 58,205 | 2869 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 30 | 58,475 | 2884 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 31 | 59,419 | 2941 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |
| 32 | 60,074 | 2981 | nd | Disaccharides | C ₁₂ H ₂₂ O ₁₁ | 342 |

To test the use of primary metabolite root exudates as TEAs and hence evidence new anaerobic respirations, we selected among identified primary metabolites (Table 1): 6 sugars (fructose, 4 disaccharides (D-cellobiose, D-gentiobiose, melibiose and maltose), and mannitol, a substrate similar to sorbitol), 2 organic acids (malic acid and citric acid) and 2 amino acids (L-serine and L-alanine).

3.3 *A. fabrum* C58 is able to use some plant primary metabolites as TEAs

To evidence the use of certain C and N sources as TEAs by *A. fabrum* C58, we applied the screening method set up previously. Sixteen C and N sources were chosen: 10 substrates from identified maize primary metabolite root exudates (Table 1) and 6 substrates (xylose, rhamnose, fumarate, succinate, leucine, aspartate) not identified in our study but known to be exuded by maize plant (Fan et al., 2012).

Controls ensuring anaerobiosis and anaerobic respiration establishment previously set up were performed. To evidence the use of substrates as TEAs, we compared growth on glucose and on each tested substrate. As seen previously, no growth was observed on glucose as TEAs (Fig. 4). Relative to the glucose control, significant growth was observed on 3 sugars, 3 carboxylic acids and 3 amino acids (Fig. 4). In descending order, the magnitude of *A. fabrum* C58 growth was as follows: xylose/fumaric acid > fructose/malic acid/succinic acid/L-serine > mannitol >> L-alanine. No growth was observed on maltose, rhamnose, D-cellobiose, gentiobiose, melibiose, citric acid, aspartate and leucine (Fig. 4). The growth of *A. fabrum* C58 on xylose, fructose, mannitol, fumaric acid, malic acid, succinic acid, L-serine and L-alanine suggests their use as TEAs for anaerobic respiration.

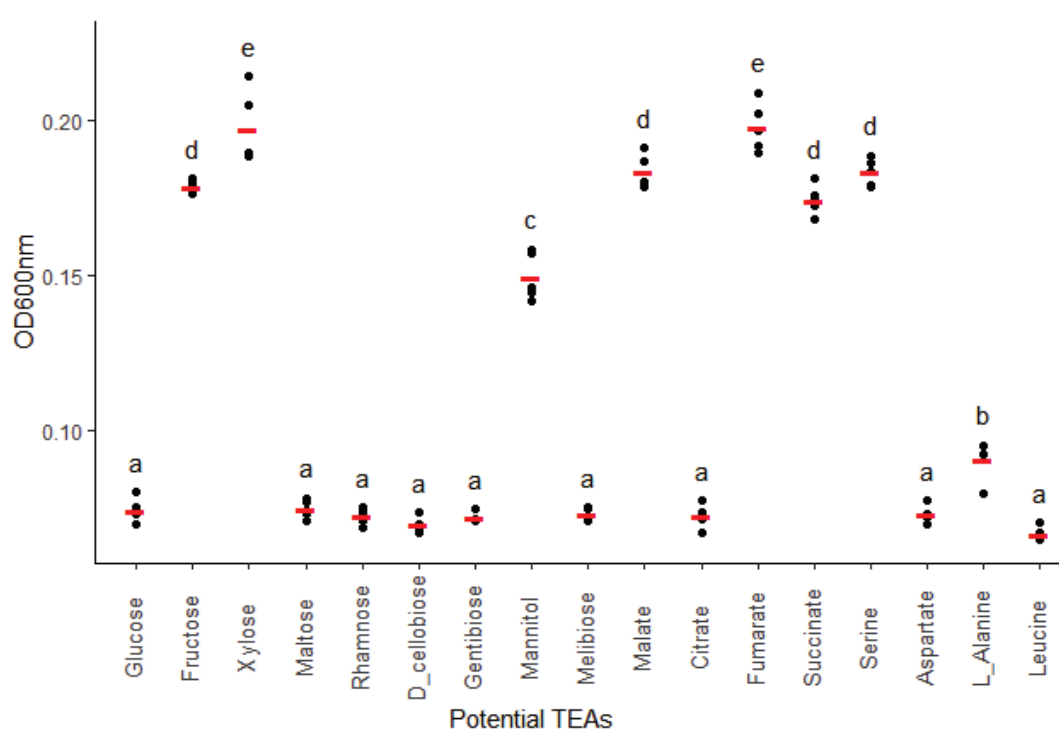


Figure 1. Growth of *A. fabrum* C58 on carbon and nitrogen sources as TEAs in microplates under anoxic conditions. Microplate manipulations were performed in anoxic glovebox. AT minimum medium supplemented with glucose and AT minimum medium supplemented with glucose and a potential TEA (xylose, fructose, maltose, rhamnose, D-cellobiose, gentiobiose, melibiose, mannitol, malic acid, fumaric acid, succinic acid, citric acid, aspartic acid, L-serine, L-alanine, and leucine) were inoculated with *A. fabrum* C58 WT. Growth of this strain was measured after 1 week at 28°C under anoxic conditions using spectrophotometer (TECAN). All experiments were performed in 5 replicates. Significant difference with AT glucose is figured by letters.

Interestingly, fumaric acid exuded by various plant species such as maize, banana, barley and *D. glomerata* (Jousset et al., 2014; Zhang et al., 2014b; Guyonnet et al., 2017) is used by *A. fabrum* C58 for anaerobic respiration. Fumaric acid respiration has been described only in a few bacteria, such as *E. coli*, *Wolinella succinogenes* and *Shewanella* (Ackrell, 1992; Kröger et al., 2002; Arkhipova & Akimenko, 2005). Fumarate is reduced to succinate, the reverse reaction of succinate to fumarate that occurs aerobically in the tricarboxylic acid cycle (TCA cycle). We evidenced, for the first time, that fumaric acid respiration may occur in *A. fabrum* C58 rhizobacteria and probably in plant rhizosphere. The anaerobic respiration of fumarate in succinate with glucose as electron donor produce enough energy to sustain growth ($\Delta G = -240$ kcal / mol of glucose): fumarate + 2 glucose \rightarrow 2 CO₂ + succinate.

Sugars, such as fructose and xylose, are also used as TEAs by *A. fabrum* C58 rhizobacteria. These anaerobic respirations are not due to fermentative process as *A. fabrum* C58 lacks fermentative genes (Lassalle et al., 2017). Xylose is the second most abundant sugar present in plant lignocellulose after glucose and is frequently found in root exudates (Jones et al., 2004; Vranova et al., 2013). Fructose is also often found in root exudates of plants, such as *Fagus sylvatica* (Zang et al., 2014), potato (Hoysted et al., 2018) and *A. thaliana* (Carvalhais et al., 2015).

A. fabrum C58 is able to colonize efficiently maize rhizosphere (Chèneby et al., 2004). Remarkably, mobility tests demonstrated a highly sensitive chemotaxis system towards sugars exuded by maize in *A. tumefaciens* C58C1 (Loake et al., 1988). Among highly chemoattractant sugars, sucrose, glucose and fructose are recognized by this strain. Collectively, these results suggest that *A. fabrum* C58 could have adapted its chemotaxis system to improve its response to plant root exuded sugars to efficiently colonize rhizosphere. Diversifying the use of carbon-containing and nitrogen-containing exudates through anaerobic respiration, could be considered as a rhizospheric trait allowing competitive advantage as shown for denitrification (Philippot et al., 1995).

In the presence of multiple TEAs in a complex environment, such as the rhizosphere, microorganisms will use TEAs in specific order (Achtnich et al., 1995). Even if this order is specific to each microorganism, they generally prefer to use the more energetic option first (Achtnich et al., 1995). The most energetic anaerobic respiration for rhizobacteria is denitrification (Lecomte et al., 2018, Table 2). However, soil nitrate spatial and temporal availability is highly heterogeneous (Arth & Frenzel, 2000), and plants compete for nitrate uptake with nitrate-reducing bacteria (Moreau et al.,

2015). In nitrate limiting environment, rhizobacteria may adapt and diversify their anaerobic respiration for growth or cell maintenance by using alternative TEAs, such as primary metabolite root exudates.

It is well-known that the free energy released during the electron transfer process in denitrification is more favorable for bacterial growth than that in fumaric acid respiration (Thauer et al., 1989; Yoon et al., 2013). Exploring enzyme kinetics of each identified TEA will determine the energy produced during these anaerobic respirations and hence give clues on anaerobic respiration role in growth and/or cells maintenance.

For the first time, this study highlights that rhizobacteria such as *A. fabrum* C58 is able to perform anaerobic respiration using TEAs identified as primary metabolites exuded by maize roots. Better understanding of these anaerobic respiration pathways is needed, especially mechanisms and genes encoding these processes and their ecological role in rhizosphere. One way to evidence these genes is mutagenesis combined with high sequencing provided by transposon sequencing (Tn-seq) (Royet et al., 2018; Gonzalez-Mula et al., 2019). Growing Tn-seq library, already constructed (Lecomte et al., Chapter 2), on individual identified TEA in anaerobic flasks will reveal which genes are crucial to respire anaerobically this substrate. Performing growth competition assays between mutant of these genes and WT strain will improve our comprehension of ecological role of these anaerobic respirations in rhizosphere.

Acknowledgments

We knowledge funding from “La Fédération de Recherche (FR) BioEnviS, Université Lyon 1” and the French National Research Agency (ANR-18-CE32-0005, DIORE). S. Lecomte received doctoral grant from the French Ministère de l’Education nationale de l’Enseignement Supérieur et de la Recherche. Plants were grown at the greenhouse platform (FR BioEnviS, University Lyon 1). GC-MS analysis was performed at the CESN platform (UMR 5557, University Lyon 1). We knowledge D. Abrouk from Ibio platform (UMR 5557, University Lyon 1) and J. Trumello (UMR7265, CEA Cadarache) for their kind help for statistical analysis.

Authors contributions

SL, XN and FZH set up the experiment. SL performed experiments, AM performed set up experiments. GM performed GC-MS analysis and metabolite annotation. PO provide advices for anaerobic experiment in glovebox. SL and FZH wrote the paper and GM, PO and XN reviewed and approved the final manuscript.

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Discussion & perspectives

1 Context and hypothesis

Agrobacterium genus lives in different habitats (soil, rhizosphere, host plants), and hence face different environmental constraints, such as C and N substrate availability, oxygen limitation etc (Dessaux & Faure, 2018). *Agrobacterium* is organized in populations and several strains and genomic species are commonly found in the same ecological niches (Costechareyre et al., 2010). Because complete competitors cannot coexist, co-occurring species must be adapted to partly different ecological niches. This co-occurrence of *Agrobacterium* strains may be allowed by species-specific genes (Lassalle et al., 2011). *Agrobacterium* harboring virulence genes carried by the Ti plasmid are able to modify the genome of their host and to construct a specific niche, i.e. the plant tumor. To colonize the rhizosphere and tumor environments, *Agrobacterium* have evolved the capacity to exploit diverse resources, such as opines (Dessaux & Faure, 2018), and probably to respire under micro-oxic condition largely encountered in these niches. In agrobacteria, denitrification, the anaerobic respiration of nitrogen oxides, is the only one validated anaerobic respiration. Among *Agrobacterium* genus, *A. fabrum* C58 is a partial denitrifier that lacks the NosZ reductase (Baek & Shapleigh, 2005). The ability to denitrify is not found in all species of *Agrobacterium* partially explaining their distribution in different habitats.

The hypothesis of this thesis was **that anaerobic respiration diversification, notably denitrification, could at least partly explain the coexistence of Agrobacteria and their distribution in specific niches in the rhizosphere.** In order to test this hypothesis, we need first to better characterize denitrification respiration and to evidence whether other anaerobic respirations may occur in the rhizosphere. We proposed in this thesis to use first *A. fabrum* C58 as model strain (member of *Agrobacterium tumefaciens* species complex). The objectives of this thesis were to (1) complete the characterization of denitrification pathway in *A. fabrum* C58, (2) explore denitrification role in *A. fabrum* C58 fitness and root colonization and (3) discover new anaerobic respirations that may occur in the rhizosphere of plants (**Figure 1**).

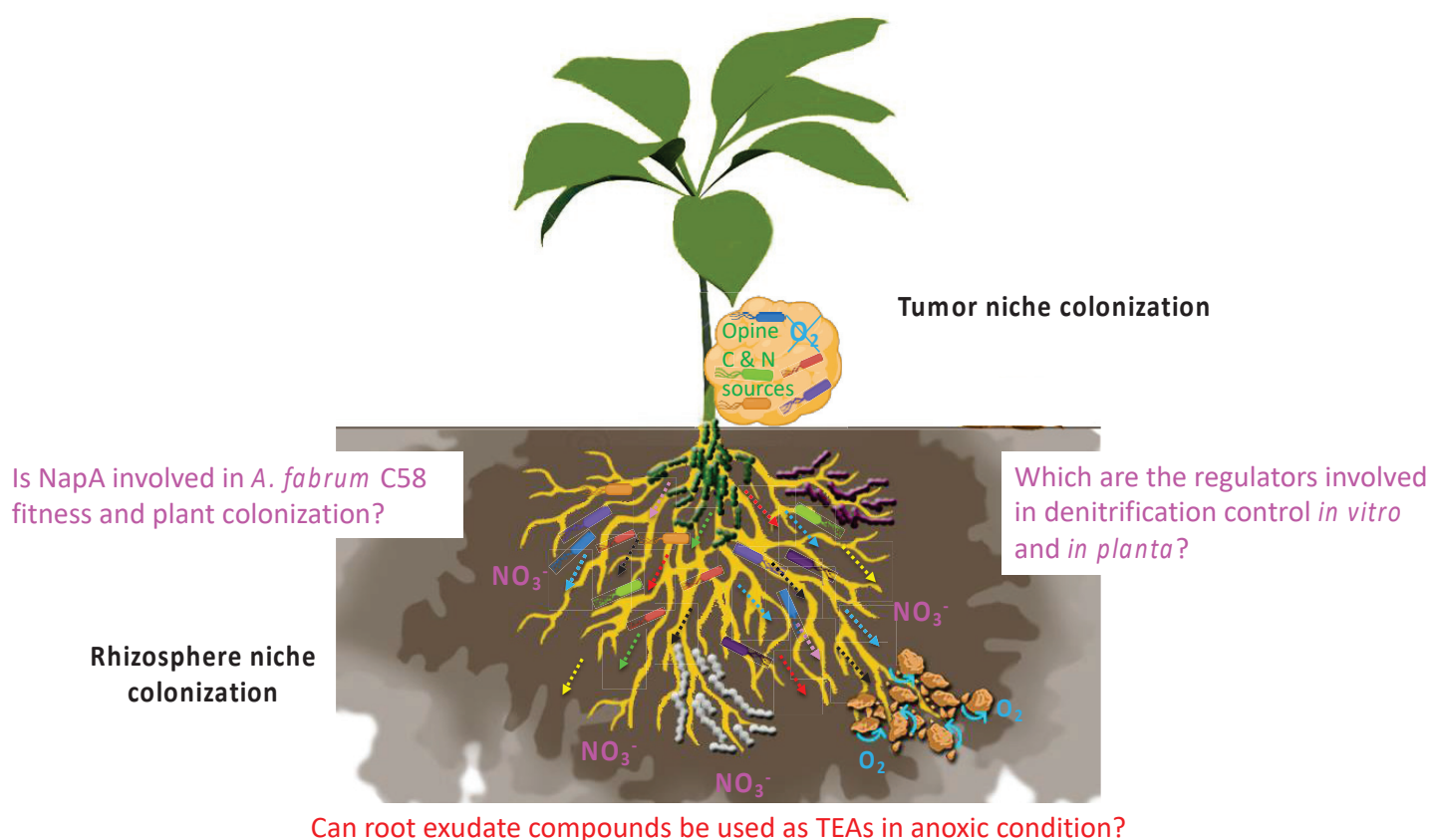


Figure 2. Hypothesis and objectives

2 Rhizosphere niche exploitation

2.1 Denitrification characterization in *A. fabrum* C58

2.1.1 NapA is a nitrate reductase involved in *A. fabrum* C58 fitness

Several studies have demonstrated that denitrifying bacteria predominantly occur near or inside the roots (Clays-Josserand et al., 1999; Chèneby et al., 2004) and proposed that denitrification is a rhizospheric trait providing a significant advantage for root colonization (Ghirardi et al., 2012). Denitrification is a respiratory process in which nitrate is reduced stepwise to dinitrogen ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$) (Zumft, 1997). The first step is catalyzed by dissimilatory nitrate reductase. Two types of nitrate reductase have been found and differ in their locations: a membrane-bound (Nar) and a periplasmic (Nap) nitrate reductase (Moreno-Vivián et al., 1999). In the rhizosphere, the membrane-bound nitrate reductase is present in *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Archaea*, whereas the periplasmic nitrate reductase is present only in *Proteobacteria* (Philippot & Højberg, 1999; Richardson, 2000; Bru et al., 2007), however some exception exist (Bedzyk et al., 1999). There are two systems responsible for dissimilatory nitrate reduction in denitrifiers (Bedzyk et al., 1999). The first system, represented by *P. denitrificans*, uses the membrane-bound nitrate reductase as the primary enzyme for nitrate reduction, while the periplasmic enzyme has only secondary

functions, including aerobic denitrification (Bell et al., 1993), transition to anaerobic respiration, or dissipating excess reducing equivalents (Potte et al., 2001). In this system like in aerobic respiration, the reaction chain is split over the periplasmic and cytoplasmic compartment, and it allows for the generation of a proton motive force across the bacterial membrane, which is exploited for ATP synthesis (Kraft et al., 2011). Because of ATP generation, Nar, the membrane-bound nitrate reductase, has been considered for a long time as the sole enzyme involved in dissimilatory nitrate reduction. In the second system, represented by strain *Pseudomonas sp.* G-179, the periplasmic nitrate reductase is required for the first step of denitrification. The status and function of the membrane-bound nitrate reductase in this system are unknown. In addition, the generation of energy provided by Nap has not been yet elucidated. In our study, we evidenced that *A. fabrum* C58 possesses the second system and that Nap is the nitrate reductase responsible for the first step of denitrification. Indeed, the *napA* mutant strain was completely disrupted in nitrate reduction and unable to grow under anoxia. However, this strain was able to denitrify with nitrite as TEAs. This result was also observed with *Bradyrhizobium japonicum* strain (Bedmar et al., 2005) suggesting the role of Nap in *A. fabrum* C58 fitness. Interestingly, during competition assay, we demonstrated that C58 Δ *napA* was able to grow poorly in presence of WT strain. We proposed a Black Queen Hypothesis (BQH) context in which denitrification function could structure the *Agrobacterium* populations in different ecological niches.

The role of nitrate reductase in rhizosphere colonization have been investigated in several species (Ghiglione et al., 2000; Mirleau et al., 2001). All reported studies were conducted on strains harboring membrane-bound nitrate reductase and no data are available for strains harboring periplasmic nitrate reductase. These studies demonstrated that Nar deficient strains were affected in competitiveness but considering the broad taxonomic and functional diversity of nitrate-dissimilating bacteria, generalization to other bacteria naturally present in the rhizosphere remains to be investigated. Ghiglione et al. (2000) reported that in *Pseudomonas fluorescens* YT101, the presence of a functional structural gene encoding the respiratory nitrate reductase confers higher rhizosphere competence. Mirleau et al. (2001) demonstrated that the nitrate reductase gives to *P. fluorescens* C7R12 a selective advantage. They suggested that the ability of *P. fluorescens* C7R12 to switch from aerobic respiration to anaerobic respiration would account for its ability to remain competent under various soil environment conditions. On the contrary, Rediers et al. (2009) observed in *P. stutzeri* A15 that a *narG* mutant better colonized roots than the wild type, which was outcompeted by the mutant strain. The role of *A. fabrum* C58 periplasmic nitrate reductase in rhizospheric colonization of maize roots has not been evidenced. Indeed, the C58 Δ *napA* strain was able to grow and colonize maize roots under anoxic conditions suggesting that the ability to respire other rhizospheric compounds could contribute to rhizosphere competence.

It has also shown that Nir's reduction of nitrites offers a competitive advantage in *Pseudomonas* strain (Philippot et al., 1995). In *A. fabrum* C58, the inactivation of *nirK* gene had no significant effect on the ability of this strain to bind to *Arabidopsis thaliana* roots under anoxic conditions (Baek & Shapleigh, 2005). However, we believe that *A. fabrum* C58 could produce NO in the presence of rhizospheric competitors and/or use NO-derived plant to colonize efficiently plant roots. Further studies are needed to understand the ecological role of denitrification genes of *A. fabrum* C58 in the rhizosphere environment.

2.1.2 Role of non-coding RNA in denitrification regulation

Small regulatory RNAs (sRNAs) are appropriate for denitrification fine-tuned regulation because they play critical roles in many physiological and adaptive responses in bacteria (Harfouche et al., 2015). A large number of potential sRNAs have been discovered through the use of high-throughput RNA-sequencing approaches in different rhizospheric bacteria (Harfouche et al., 2015). Evidences indicated that they control biological functions required for both beneficial and deleterious plant-bacteria interactions. In *Bradyrhizobium*, *Sinorhizobium meliloti* and *Rhizobium elti*, sRNA are involved in symbiosis (Madhugiri et al., 2012; Torres-Quesada et al., 2010; Vercruysse et al., 2010). On the contrary, in *Erwinia amylovora*, *Xanthomonas campestris* and *A. tumefaciens* plant pathogen, they are involved in the virulence control (Zeng et al., 2013; Chen et al., 2011; Wilms et al., 2012; Leonard et al., 2017). Recently, QfsR, a plasmid-encoded sRNA, has been demonstrated to control chromosomal gene expression involved in flagellar apparatus in *A. fabrum* C58 (Diel et al., 2019). We demonstrated that NopR sRNA is involved in denitrification regulation. We suggest that under anoxic condition, NopR confers increased stability to *norQ* mRNA allowing the production of maturation protein NorQ, inducing NorC activation and consequently an increased in N₂O emission. Even if we do not have a clear demonstration of the increase of NO consumption by C58 NopR⁺ strain, we suggest that NopR sRNA regulates NO level. We hypothesize that NopR sRNA could be a way to counteract the NO-derived from plants (involved in plant defense) in order to better colonize the host plant.

To evidence and understand the ecological role of *A. fabrum* C58 sRNAs in root colonization and virulence, high-throughput RNA sequencing performed by Dequivre et al. (2015) and Gonzalez-Mula et al. (2018) could be combined.

2.1.3 *A. fabrum* C58 key genes for denitrification

Transposon sequencing (Tn-seq) is an attractive approach used in different rhizobacteria to determine key genes involved in an ecological pathway. For examples, in *Dickeya dadantii*, Tn-seq were used to identify the genes required for the multiplication in chicory (Royet et al., 2018). In *Pseudomonas* sp. WCS365, Tn-seq library inoculated on *A. thaliana* roots reveals genes required for

both rhizosphere fitness and for evading host immune responses (Liu et al., 2018). Recently, Gonzalez-Mula et al. (2019), in an elegant study, combined plant metabolomics approach and two functional genome-wide scans (transcriptomics and Tn-seq) to identifying the genes and pathways involved in the exploitation of the *Solanum lycopersicum* host by *Agrobacterium tumefaciens* C58. However, to our knowledge, Tn-seq approach was never been applied under anoxic conditions to identify genes involved in denitrification regulation and their role in root colonization.

Tn-seq has been qualified as a “relatively simple” approach (Gonzalez-Mula et al., 2019). However, we had to consider three limitations of Tn-seq analysis to apply it to our restrictive anoxic condition and several adjustments were made. Firstly, we must ensure the number of generations needed for the Tn-seq experiment. Growth in anoxia is limited due to low energy generation. To overcome this, we had to increase the number of *in vitro* and *in planta* repetitions.

Secondly, in stepwise pathway as denitrification, transposon mutants could use the product of other mutants to their own benefit and thus counteract their mutational insertion. We used nitrate and nitrite as TEAs to obtain key genes involved in these steps of denitrification.

Thirdly, during host colonization, Tn-seq analysis required an outgrowth step after the recovery of bacteria from roots (Wilde et al., 2015). To avoid confounding effects on fitness calculations, we had to adapt the outgrowth time.

After sequence analysis, mutants will be constructed, and their fitness and role in root colonization will be evaluated.

2.2 Diversification of anaerobic respiration in *A. fabrum* C58

In the rhizosphere, the roots of growing plants modify the partial pressure of oxygen in the soil, in their immediate vicinity (Kuzyakov & Razavi, 2019). This variation in oxygen concentration constrains bacteria to switch from an aerobic metabolism to an anaerobic one. Bacteria have evolved the capacity to respire in anoxia, such as denitrification (Richardson, 2000; Lecomte et al., 2018). Denitrification is the most energetic anaerobic respiration (Thauer et al., 1989). However, plants and bacteria compete for nitrate which is not always available in the rhizosphere (Moreau et al., 2015). We hypothesized that bacteria have diversified their anaerobic respiration through the use of available rhizospheric compounds as TEAs to survive and/or grow in the vicinity of roots. Among the available rhizospheric compounds, we have hypothesized that primary metabolites (sugars, amino acids and organic acids) exuded by plant roots are respired anaerobically by rhizobacteria giving them a competitive advantage for root colonization.

Among putative TEAs in rhizosphere, fumaric acid, largely exuded by plants (Guyonnet et al., 2017), has been already characterized for anaerobic respiration of *E. coli*, *W. succinogenes* and

Shewanella (Ackrell, 1992; Kröger et al., 2002; Arkhipova & Akimenko, 2005). Fumaric acid is reduced to succinic acid, the reverse reaction of succinic acid to fumaric acid that occurs aerobically in the tricarboxylic acid cycle (TCA cycle). However, this respiration has not been studied with model strains inhabiting the rhizosphere of plants. Rhizobacteria models and new method to screen putative TEAs are needed to discover new anaerobic respirations that may occur in the rhizosphere of plants.

2.2.1 Set up of a new screening method and its implementation with putative TEAs

We set up a new method named “TEAMS” for TEAs Method Screening that can be applied to any model strain with few adjustments to screen putative TEAs. TEAMS is based on the determination of an electron donor and an acceptor, and on the guaranty of the absence of oxygen. It is a rapid and efficient system that enables the screening of twenty C and N sources in one time with 4 replicates for each putative TEA. We screened C and N sources known to be exuded by maize plants to get closer to the rhizosphere environment. For the first time, we demonstrated that *A. fabrum* C58 was able to perform anaerobic respiration using D-xylose, mannitol, D-fructose, fumaric acid, malic acid, succinic acid, L-serine, and L-alanine as TEAs.

2.2.2 How to get insights on the characterization of anaerobic respiration?

Among identified TEAs, we could only determine the energy generated by the anaerobic respiration of fumaric acid. Fumaric acid respiration generates -240 kcal/mol of glucose. This value suggests that fumaric acid respiration may sustain *A. fabrum* C58 growth. To determine the energy yield during anaerobic respiration using D-xylose, mannitol, D-fructose, malic acid, succinic acid, L-serine, and L-alanine as TEAs we need to know the product of these respirations. To do so, we could use ¹³C-enriched TEAs. Their reduction by *A. fabrum* C58 during anaerobic respiration will lead to the apparition of ¹³C-enriched product that can be identified and hence used to calculate the free energy generated. These values will give clues on the role of each respiration to sustain and/or maintain bacterial growth under anoxic conditions.

The Tn-seq approach have been set up for the identification of denitrification genes (number of generations, number of replicates). This experiment served as a proof of concept for the characterization of anaerobic respiration pathways. We proposed to use the transposon mutant library to reveal the key-genes, i.e. reductases and regulators, involved in anaerobic respiration of fumaric acid, D-xylose, mannitol, D-fructose, malic acid, succinic acid, L-serine, and L-alanine.

We demonstrated in Chapter 1 that denitrification genes are not harbored by all *A. tumefaciens* complex species. It is questionable whether new anaerobic respirations are also species-specific

pathways related to genome diversity. To determine the specificity of each pathway, TEAMS could be applied to *A. tumefaciens* spp. We expect to see differences in growth rapidity and/or efficiency that could explain the distribution, structuration and co-occurrence of *Agrobacterium tumefaciens* spp. in rhizosphere.

3 Tumor niche exploitation

Tumor is a constructed niche where agrobacteria proliferate through metabolic adaptation, such as opine degradation, osmoprotection establishment and oxidative stress resistance (Dessaux & Faure, 2018; Meyer et al., 2019). Deeken et al. (2006) investigated plant metabolism occurring in tumor and thus provided evidence that tumors are deprived of oxygen. Agrobacteria have already adapted their metabolism to reduce opine produced in tumor and thus take advantages on other bacteria unable to use opines as carbon and nitrogen source. Agrobacteria may evolve the capacity to shift from oxic respiration to anoxic respiration to colonize tumor environment. We propose to investigate the competitive advantage given by denitrification genes in tumor colonization and bacterial survival and/or maintenance in this environment.

Gonzalez-Mula et al. (2018) evidenced in tumoral environment an increase of NO reductase *norCB* expression, but neither that of nitrate reductase *napA*, nor that of nitrite reductase *nirK*. They suggest that NO only is sensed by *A. tumefaciens* in plant tumors and Nor production could be a way to counteract NO accumulation in plant and beyond 'to manipulate' NO-regulated plant defense reactions (Gonzalez et al., 2018). However, no information was provided on the level of O₂ and nitrate in tumor environment. To determine the role of denitrification genes in tumors colonization, we propose to monitor denitrification genes expression and in the same time to measure O₂ level and nitrate concentration.

Metabolomics of tumor tissues induced on *S. lycopersicum* by *A. tumefaciens* revealed a wide variety of metabolites (e.g. sugars, polyols, amino acids, organic acids, phenolics) present at a quite similar concentration in uninfected stems (Gonzalez-Mula et al., 2019). These plant metabolites could support bacterial growth when *A. tumefaciens* colonizes either asymptomatic or symptomatic plants. Interestingly, 24 metabolites, such as γ -aminobutyric acid (GABA), proline, pyruvate, γ -hydroxybutyric acid (GHB), succinic semialdehyde (SSA), and opines, were enriched at least 4 times in plant tumors compared with healthy stems. *A. tumefaciens* was expected to have evolved pathways for detoxifying and exploiting these enriched compounds as nutrients and signals (Gonzalez-Mula et al., 2019). We also proposed that agrobacteria have evolved anaerobic pathways for respiring these enriched compounds. To test this hypothesis, we could combine metabolomic data with TEAMS and Tn-seq analysis. Compounds revealed by Gonzalez-Mula et al. (2019) could be screened in anoxia using

TEAMS. Tn-seq analysis could be performed in anoxia in the presence of tumor compounds previously identified to be respired. These experiments will improve our comprehension of the role of anaerobic respiration diversification in survival and/or cell maintenance in tumor environment.

We demonstrated in *A. fabrum* C58 that (1) Nap is involved in the fitness but not in the root colonization, (2) NopR is involved in denitrification control and (3) this model strain is able to anaerobically respire 7 compounds known to be exuded by maize roots (**Figure 2**). Our hypothesis was that anaerobic respiration diversification is a worthy explanation for the coexistence of *Agrobacteria* and their distribution in specific niches in the rhizosphere and tumor environments. To entirely validate this hypothesis, other *Agrobacterium tumefaciens* strains need to be tested. TEAMS, Tn-seq approach and rhizosphere colonization experiments set up on *A. fabrum* C58 have now to be applied to these strains.

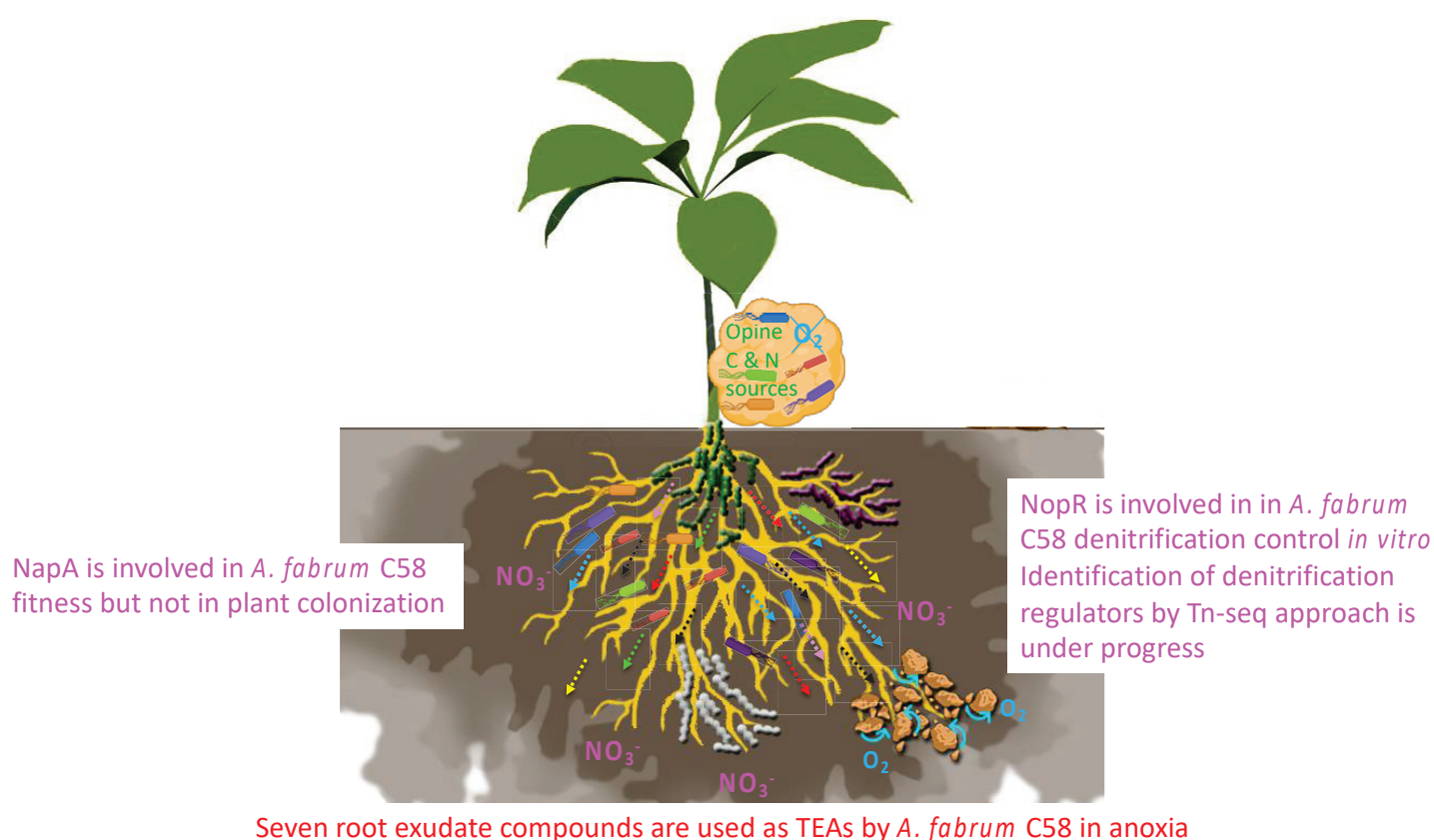


Figure 3. Conclusions

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