PEOPLE‟S DEMOCRARIC REPUBLIC OF ALGERIA
وزارة التعليم العالي والبحث العلمي
MINISTRY OF HIGHER EDUCATION AND SCIENTIFIC RESEARCH
جامعة عمار ثليجي الأغواط
AMAR TELIDJI UNIVERSITY OF LAGHOUAT
كلية العلوم
FACULTY OF SCIENCES
قسم البيولوجيا
DEPARTEMENT OF BIOLOGY

In view of obtaining MASTER degree

Field: BIOLOGICAL SCIENCES

Option: BASIC AND APPLIED MICROBIOLOGY

Theme

Identification and study of endophytic bacteria in the model plant *Medicago truncatula*

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Sustained on: 01/07/2023

Summery

Acknowledgement

First of all, I thank God, Almighty, for having me given the strength, as well as the audacity to overcome all the difficulties.

I address my most sincere thanks to my supervisor **Dr. Berrabah Fathi**. Who guided, supervised the progress and execution of the work of this thesis by providing me with all possible help, and by devoting a lot of his precious time to me, his knowledge and expertise were invaluable

I am grateful to my co-promoter **Pr. Gruber Véronique** for her scientific collaboration and all the resources and support she provided.

I would like to thank **Pr. Rachid Chaibi** , **Dr. Benaceur Farouk** and **Dr. Rezzoug Asma** for their interest in my work by being a member of the jury and providing valuable feedback and suggestions

Finally, I would like to express my sincere gratitude to all the participants in my study. Their willingness to share their experiences and insights was invaluable to my research and contributed to the success of this research. Thank you for your time and contribution

Dedication

I dedicate this work to those who are very dear to me, and without whom I would never have reached the stage where I am today To my dear parents **Fatiha** and **Mohammed**

No dedication can express my respect, my love and my consideration for the sacrifices you have made for my education and my well-being. May this work be a testimony of my gratitude and my sincere and faithful love.

To my sister **Nesrine** and my brothers **Islam** and **Abd el raouf,** words are not enough to express the attachment, the love that I have for you

Abstract

In this study, the effects of endophytes on the *Medicago-Sinorhizobium* symbiosis and the interaction between *Medicago* and endophyte bacteria were studied. For this, soil from organic farms or the desert was used to create a collection of endophytic bacterial strains. The capacity of 46 of them to infect *Medicago* R108 and A17 roots under axenic conditions was investigated. Five strains (M17, M18, M50, M67.2 and BT-37) among the forty validated bacteria were selected for molecular identification .We studied the potential effects of the PGPR of the strain studied, the analysis of the response of Medicago to salt stress in the presence or in the absence of endophytes was evaluated. The results indicate the presence of various effects of the tested strain on the protection of *Medicago* against abiotic stress. Interestingly, the less infectious strain may possess the highest protection against salt stress.
the qPCR analysis of the *PR10, PR5* and *NDR1-Like* genes after inoculation of the A17 ecotype by the endophytes showed Different levels of immune stimulation are observed depending on the strain used. Interestingly, the expressions of the *PR10* genes are correlated with the level of tissue occupancy by endophytes .Finally, we have studied the impact of endophytes on the *Medicago-Sinorhizobium* symbiosis, a co-inoculation of *Medicago* with the strain studied and the *rhizobium* was carried out. Our preliminary data suggest that endophytes have a weak impact on the initiation of the symbiotic interaction. endophytes tested are able to infect nodules and reduce nitrogen-fixing capacity. Interestingly, *Lysinibaccillus sp*. These show the lowest root colonization ability, display the highest nodule infection rates. This observation underlies the difference between nodule and roots and could be explained by the difference in immunity and/or metabolic status. Finally we were able to demonstrate that infection rates do not seem to be correlated with nitrogen fixation

Résumé

Dans cette étude, les effets des endophytes sur la symbiose *Medicago-Sinorhizobium* et l'interaction entre Medicago et les bactéries endophytes ont été étudiés. Pour cela, des sols issus de fermes biologiques et du désert ont été utilisés pour créer une collection de souches bactériennes endophytes. La capacité de 46 d'entre eux à infecter les racines de *Medicago* R108 et A17 dans des conditions axéniques a été étudiée. Cinq souches (M17, M18, M50, M67.2 et BT-37) parmi la quarantaine de bactéries validées ont été sélectionnées pour l'identification moléculaire nous avons étudié les effets potentiels du PGPR de la souche étudiée, l'analyse de la réponse de Medicago au stress salin en présence ou en l'absence d'endophytes a été évaluée. Les résultats indiquent la présence de divers effets de la souche testée sur la protection de *Medicago* contre le stress abiotique. Fait intéressant, la souche la moins infectieuse peut posséder la protection la plus élevée contre le stress salin.
L'analyse qPCR des gènes *PR10, PR5* et *NDR1-Like* après inoculation de l'écotype A17 par les endophytes a montré Différents niveaux de stimulation immunitaire sont observés selon la souche utilisée. De manière intéressante, les expressions des gènes *PR10* sont corrélées au niveau d'occupation tissulaire par les endophytes Enfin, nous avons étudié l'impact des endophytes sur la symbiose *Medicago-Sinorhizobium*, une co-inoculation de *Medicago* avec la souche étudiée et le *rhizobium* a été réalisée . Nos données préliminaires suggèrent que les endophytes ont un faible impact sur l'initiation de l'interaction symbiotique. les endophytes testés sont capables d'infecter les nodules et de réduire la capacité de fixation de l'azote. Fait intéressant, *Lysinibaccillus sp*. Ceux-ci montrent la capacité de colonisation des racines la plus faible, affichent les taux d'infection des nodules les plus élevés. Cette observation sous-tend la différence entre le nodule et les racines et pourrait s'expliquer par la différence d'immunité et/ou de statut métabolique. Enfin nous avons pu démontrer que les taux d'infection ne semblent pas être corrélés avec la fixation d'azote

Parte I. Bibliographic Synthesis

Chapter I. Introduction to plant-microbes interactions

I. The plants are surrounded by thousands of microbes

Plants are surrounded by high density of microbes and the roots remain the more exposed organ due to its location (into the soil). The rhizosphere connects the roots to the soil, this area is a highly dynamic since the roots interact with a wide range of microbes, including pathogenic, beneficial and commensal. Some of the most complex biological interactions experienced by terrestrial plants are those that exist between roots and their surrounding environment of soil [1].

Depending to their location into the roots, two types of microbes can be defined: the first called epiphytes colonized the roots surface and the second termed endophytes are able to penetrate inside organ and colonized roots tissues [2]. In the aim to promote beneficial microbes and to prevent pathogens, the plants control the microbial composition of the rhizosphere by secretion of root exudates. The exudates comprise high molecule diversity, including low-molecular weight compounds (e.g., sugars, amino acids, organic acids, nucleotides, peptides, inorganics, and hormones) and high-molecular-weight compounds (e.g., polysaccharides and proteins[3]). The secondary metabolites of the roots exudate are chemotactants, involved in the attraction of beneficial microbe. The roots exudates are produced by specialized border cells (SBC) released by the roots apex during roots penetration in the soil [4]. SBC remains metabolically active weeks after the separation from the plants. The release of organic matter by the plants provides the driving force for the development of active microbial populations in the rhizosphere [1]. This phenomenon is called the rhizosphere effect. On the other hand, cohabiting microorganisms have a direct impact on plant metabolism and physiology [1].

II. Different effects are produced by microbes on the plants

Depending to their outputs, three categories of microbes can be distinguished: (i) neutral

microbe don‟t producing any effect on the plants, (ii) negative microbe represented by pathogen which altering the plant growth and (iii) beneficial microbes helping the plants by allowing nutrients, protection and stress tolerance [5]. The plants produce responses according to the interaction types: don‟t over reacts to neutral microbes, produce defences response to

pathogen and host beneficial microbes [6]. The mechanisms involved in the plants response to microbes involved complex and well organized genetic network.

III. Molecular mechanisms controlling the plant responses to microbes

The plants perceive microbes‟ trough specialized receptors recognizing microbial compounds (ligand). After the binding of the ligand to the receptor, intracellular singling event occur and leads to the activation of genes expression and physiological responses [7]. Depending to the type of microbes (pathogens or beneficial), the plant produce distinct responses.

III.1. Plant response to pathogens

Root exudates can attract beneficial organisms, but they can also be of interest to pathogenic populations, many of which express virulence on a limited number of host species [8]. Pathogenic microbes have coexisted with plants and have a high degree of host specificity [9]. Diseases caused by phytopathogenic (plant pathogens) microbes affect all food-producing plants and can lead to the suppression of the infected plants [10]. During their co-evolution, plants and pathogenic microorganisms have developed complex relationships resulting from a constant exchange of molecular information. Pathogens have evolved a range of offensive strategies to parasitize plants and in return, plants have deployed a similar defensive arsenal [11–13].

III.1.A. PAMP Triggered Immunity

The PAMP Triggered Immunity (PTI) is the first level of plant defence and lead from low to moderate immune response. PTI is activated by Pattern Recognition Receptors (PRRs) localized in the cytoplasmic membrane and detecting conserved compounds of microorganisms, the Pathogen/Microbe-Associated Molecular Patterns (P/MAMPs). Binding of the P/MAMPs to the PRRs lead to activation of defence signalling, which include the phosphorylation of specific protein such as the Mitogen Associated Protein Kinase (MAPK), the influx of extracellular calcium inside the cell and the production of Reactive Oxygen Species (ROS) such as hydrogen peroxide or nitric oxide [7,8,14]. The defence singling leads to secretion of antimicrobial compounds [7], biosynthesis of defence hormone and up-regulation of defence genes such as the *Pathogenesis Related protein 10* (*PR10*, [15]) and *PR5* [16] encoding respectively a endonuclease [17] and thaumatin-like protein [18]. PR10 control the cell death [19] and PR5 display antimicrobial properties [16].

III.1.B. Effector Triggered Immunity

To avoid the PTI, phytopathogenic bacteria and fungi produce effectors [20], corresponding to small peptide injected into the host cell by the microbe. The effectors can short-circuit the defence signalling by inactivation of signalling protein [14]. Effector Triggered Sensitivity (ETS) is observed when the PTI is inactivated under the effectors effect [12].

During their evolution, the plant have been acquires Resistance (R) genes, encoding intracellular receptor called Nucleotide Binding Site (NBS) Leucine Rich Repeat (LRR) able to perceive effector [21]. Following the recognition of the effector by corresponding NBS-LRR; reactivation of the defence signalling occur and leads to Effector Triggerd Immunity (ETI, [7]). This response display a higher intensity of response compared to the PTI with ending by activation of the programmed cell death (PCD), moreover ETI is more durable and robust resistance than PTI [10].

III.1.C. Local and Systemic acquired resistance

Depending to the location of the defence response, two types of resistance can be distinguished: the Local Acquired Resistance (LAR) is activated near to the infection site and consist of cellular modification including reinforcement of the cells wall and the secretion of toxic compounds [22]. The second type is the Systemic Acquired Resistance (SAR) which is an induced defence mechanism that confers resistance in the whole plants[23]. SAR requires mobile signalling molecule such as the salicylic acid (SA) an important phytohormone that stimulate defence signalling in the plant cell [23]. The SA is associated with the accumulation of pathogenesis-related proteins [24].

IV. Introduction to plant-microbes beneficial association

In the opposite of pathogens which induce plants disease, beneficial microbes promote the plant growth and resistance [25]. Different types of beneficial association between plants and microbes were identified, depending to the fitness of the association two types of beneficial associations is observed. (i) Interaction with low fitness of association: the establishment of this interaction engage low energy by the plants; this association is produced during plant interaction with Plant Growth Promoting Rhizobacteria and Fungi (PGPR/F [26,27]). (ii) Interaction with high fitness of associations: this is observed during the mycorhize [5] and nitrogen-fixation symbiosis[25]. These symbioses consume more energy than the previous type.

The beneficial microbes have wide range of impact on the plants. Two categories of beneficial microbes effects are observed: the first is direct effects on the plant by providing macronutrient and micronutrients and by producing developmental hormones such as cytokinine and auxine [28]. Moreover the beneficial microbes can affect the stress responses by the manipulation of defence/stress hormone signalling [29]. Indirect effects represent the second type of beneficial microbial effect, this consist on the protection of the plant from the pathogen by competing with them by beneficial microbes for the plant colonization [30].

Plant-microbe symbiosis is a mutually beneficial relationship between plants and microorganisms. This interaction is crucial for the survival and growth of both parties involved. Plants provide nutrients and shelter for microbes, while microbes help plants absorb nutrients from the soil and protect them from pathogens [31]. The mycorhizia and the legume Nitrogen Fixing Symbiosis (NFS) are among the most harvested plant symbiotic interactions. The mycrohize broaden the roots area for the prospection of nutrient and water; in the addition the mycorhize provide phosphate to the host [5]. NFS is established between leguminous plant and rhizobia. This interaction is mainly giving organic nitrogen to the plant by the transformation of atmospheric nitrogen into organic form [32].

IV.1. Plant Growth Promoting Rhizobacteria

PGPR define all bacteria able to stimulate the plant growth and resistance of the plants [33]. PGPRs are distinguished by their ability to colonize the root, survive, multiply and be competitive with other microorganisms, while stimulating plant growth [26,34]. In the rhizosphere, these bacteria can be found at intra or extracellular levels [35]. At the intracellular level, the bacteria are said to be endophytic and colonize the appoplast. At the extracellular level, they are located on the surface or near the roots, so they are rhizospheric or epiphyte [2].

Several genera have been identified as containing PGPR such as *Streptomyces sp.* [36], *Bacillus sp.* [37] and *Pseudomonas sp*. [38]. Different PGPR strains produce phosphate and the potassium solubilizing enzymes , nitrogenase which convert atmospheric nitrogen N_2 in organic nitrogen form (NH_3^+). Iron chelation can be realized by PGRP thanks to the production of siderophor proteins able to bind efficiently iron [39,40].

PGPRs can also impact the growth of plants by the modulation of hormonal responses of the host. It was shown that several PGPRs strain expressed ACC dehydrogenase that‟s can

lowered the ethylene cotenant of the plants by the degradation of the precursor of the ethylene (ACC, [41]). The ethylene is a defence hormone impacting negatively the plant growth and stimulating stress-resistance genetic programme of the plants [42]. Somme PGPRs can produce developmental hormones such as cytokinine , auxines and giberline [43]. These hormones impact the plant development by affecting cell multiplication and differentiation.

Another important aspect of the PGPR role is the protection of the plant from the pathogenic microbes. Somme bacteria can produce antimicrobial compounds such as the Hydrogen Cyanide (HCN[39]) that‟s inhibiting pathogens growth. Plant defence can also be potentiated trough the recognition of MAMPs, which leads to the activation MAMP triggered Immunity (MTI [44]).

IV.2. Mycorhize

Mycorhize is the most spread symbiosis across the plant kingdom, around 80% of the plant can establish symbiosis with mycorhizial fungi. These symbiosis is proposed that‟s was actively participate on the adaptation of photosynthetic organism to the terrestrial life. Two type of mycorhize is distinguished based on the plant tissues colonization, the first type is the ectomycorhize colonizing the surface of the roots with low penetration of the plant tissues by the fungi. The second type is the endomycorhize infecting the roots at inter and intra-cellular level. These associations provide phosphate to the plants, enhance the stress protection and the area of roots prospection in the soil [5].

Chapter II. Legume-rhizobium symbiosis

I. Introduction to the nitrogen fixing symbiosis

NFS is mainly established between legumes plants such as *Medicago sp.* [25] and certain non-legume like *Parasponia sp.* [45]. NFS is established with the rhizobia, a soil nitrogen-fixing bacteria, which are gram-negative bacteria belonging to the classes of α- and β-proteobacteria living naturally free in the soil [46]. The majority of the symbiotic genes of the rhizobium are located into a plasmid, called the mega symbiotic plasmids [47] and it‟s proposed that the large diversity of the rhizobia genera is due to horizontal transmission of the NFS ability between diverse bacterial strains [48]. During legume-rhizobium symbiosis, a new roots organ is produced, the nodules hosting the rhizobium, which colonize the host cells [49].

II. Legume Nitrogen Fixation Symbiosis

The legume plant has a major advantage due to their ability to establish SNF with rhizobia. SNF allow the legume plant to grow in poor nitrogen soils [50]. This ability is an essential characteristic when the availability of nitrogen is a limiting factor for growth and is therefore of particular interest for agriculture [51].

During legume-rhizobium symbiosis, the plant develop a symbiotic organ (the nodule) hosting the rhizobium. In this organ the bacteria convert the atmospheric nitrogen (N_2) into organic form [52]. In return the plant plants provide to the bacteria a preferential ecological niche and carbon [31].

II.1. Nodule organogenesis

The symbiosis starts by a recognition phase between legume and the rhizobium. This phase occur when the nitrogen become not or less available in the soil and start by the production of specific isoflavonoids by the plant [49]. These compounds are recognized by the transcriptional regulator, NodD of the rhizobium [53]. The binding of the flavonoids to the NodD lead to the expression of the Nod operon, which containing genes encoding enzymes involved in the biosynthesis of chito-oligo-sacharids compounds, also called nodulation (Nod) factors [54]. Nod Factors (NF) bind plant symbiotic receptors and lead to the activation of symbiotic signalling [55].

After the recognition, the rhizobium invades the roots trough roots hair by formation of infection threads [56]. The thread penetrates inside the roots cell and progress toward the

roots cortex. In parallel, in the infection site, the host reactivate the division of the cortical cell giving the future nodule primordia, the first infect plants tissues [57]. NF signalling remain the most used process for legume-rhizobium recognition, however in certain legume species the bacteria can invade the host trough crack located at the junctions between primary and secondary roots [58].

Inside the nodule cell, the bacteroids (the intracellular form of the rhizobium) are encompassing into host membrane (the Peri-Bacteroids Membrane; PBM) and it‟s separated from the bacteroids by a Peri-Bacteroids Space (PBS). Symbiosome correspond to the bacteroids with PBM and PBS [59].

The bacteroids are subjected to a differentiation process which end by the expression of the bacterial nitrogenase enzyme. Nitrogenase is an enzymatic complex which catalyses the reduction of molecular nitrogen N_2 to ammonia NH_3^+ according to the following equation [52]:

$$N_2 + 8H^+ + 8^{e-} + 16\ ATP \rightarrow 2NH_3^+ + H_2 + 16\ ADP + 16Pi$$

II.2. Medicago symbiosis

II.2.A. Medicago taxonomic classification

Leguminous plants are belonging to the Fabaceae family, which are dicotyledonous plants representing the third family of plants by number of species, after Asteraceae and Orchids. Legumes are divided into three subfamilies; Caesalpinioideae, Mimosoideae, Papilionoideae (also called Faboideae), most of the cultivated species belong to this last subfamily[60].

In the Faboideae, Medicago represent a genus of approximately 87 species of herbs and shrubs widespread from the Mediterranean to central Asia includes the widely cultivated major forage crop and weedy species *Medicago sativa L* (alfalfa, lucerne) and the legume model species *Medicago truncatula* [61]. The ecotypes A17 and R108 of *M. truncatula* are the most studied Medicago genotype due to the sequencing of their genome and easy genetic manipulation [62,63]. These plants display big library of OMICs data and the availability of a large collection of plant mutants [64,65].

II.2.B. Medicago NFS specificities

Medicago produce undetermined nodule characterized by an elongated form due to the presence of an apical meristem [66]. In this nodule type, the plants tissues are organised in four zones: the first zone is the meristem (Zone I; ZI) responsible in the biogenesis of the nodule tissues. Next to the ZI, in the infection zone (ZII) the rhizobium infects the host cells and it‟s subjected to differentiation process. In the fixation zone (ZIII) the differentiated bacteroids convert the N_2 into NH_3^+. Finally when the nodule becomes old or when the plant is exposed to a stress or when organic nitrogen is supplied to the growth medium, a fourth zone (ZIV) is formed [57]. In the ZIV the bacteroids and the host cells are degraded and the cellular compounds are recycled thank to the activities of specific genes such as the Cysteine Proteases (CP, [51]).

II.2.C. Terminal Bacteroids Differentiation

Medicago is belonging to a specific subgroup of legumes called Inverted Repeat Legume Clade (IRLC, [67]). In the IRLC plant, the bacteroids are subjected to a Terminal Bacteroids Differentiation (TDB, [68]). This process end by the increase of the bacteroids seize and the genome copy number. This process is allowed thanks to the action of small antimicrobial peptides, the Nodule Cysteine Reach (NCR, [69,70]).

In the nodule, the IRLC plant produces NCRs, which are targeted to the PBS. To abolish the antimicrobial effect of the NCRs in the PBS, the bacteroids import the NCRs into its cytoplasm using specific membrane transporter such as the ATP-binding Cassette transporter BacA [71]. Inside the bacteroids, the NCRs interfere with physiological process such as the cell division, which lead to the TDB [72].

Chapter III. Endophytic microbes

I. Introduction to endophytic microbes

Endophyte defined microbes able to colonized plant tissues without induction of negative effects. They are found in many mono and dicotyledonous plants and they can be isolated from various plants tissues (roots, stems, leaves, inflorescences of weeds, fruit plants, [73]). The wide distribution of endophytes in the plants is explained by the fact that the variations in the outside environment put the plant metabolism out of homeostasis, which creates necessity for the plant to harbor some advanced genetic and metabolic mechanisms within its cellular system [74]. Herein, the importance of microbes, especially the endophytes, increases immensely.

Actually, the most studied endophytes are fungi and bacteria. Most of the endophytes fellow pathogenic behaviors and it‟s proposed that at least some of them are derived from pathogenic strains [75]. Based on the nature of pathogenicity, endophytes may be of three types: (i) pathogens of another host that are nonpathogenic in their endophytic relationship; (ii) nonpathogenic microbes; and (iii) pathogens that have been rendered nonpathogenic but still capable of colonization by selection methods or genetic alteration [76].

The relationship between host and endophytes is considered as a symbiosis with beneficiates exchanges. At present, research on endophytes mainly focuses on two aspects: the exploitation of valuable bioactive molecules produced by endophytes and the exploration of the possibility of endophytes as bio-control agents [76].

II. Plant tissues colonization by endophytes

Some studies reported a higher level of endophytes colonizing the phylloplane (plants tissues located out the soils) compared to other tissues [77]. The high nutrient content, including carbon, nitrogen, and phosphorus in the leaf tissue, may have contributed to the high microbial diversity in the phylloplane [1].

It‟s proposed that fungi and bacteria in plant roots followed a stochastic assembly process for fungi and a deterministic process for bacterial [78]. The main factors that may regulate microbial colonization include the plant genotype, the growth stage, the physiological status, the type of plant tissues, some soil environmental conditions (humidity and temperature), as

well as some agricultural practices [78]. Moreover, the colonization of plants tissues start early during the development process and it‟s greatly enhanced during senescence stages [1].

High similarity between microbes found internally in root tissue with those found in the rhizosphere has been indicated that the rhizosphere may be one of the main sources of endophytic colonization [75]. Endophyte microbes typically use two transmission patterns: vertical transmission from maternal plants into progeny seed is the primary mechanism with which the offspring is infected. The endophytes enter into newly formed plants during seed germination or can be introduced into the seed during its formation (for example into inflorescence during the fecundation stage). Several endophytes in aboveground tissues are horizontally transmitted via spores and/or hyphal fragmentation, by biotic (herbivores or insects) or abiotic dispersion agents (wind or rain) from plant to plant. Moreover the plants can acquire endophytes from the environment by *de novo* infection of roots or aerial parts by microbes [29].

Plants control the epiphyte and endophyte microbial community through multiple processes, one of the most studied is the secretion of root exudates by the host able to attract beneficial microbes for colonization of roots surface and inner tissues [3]. The second layer of control, occur during the plants selection of the microbes for penetrating their tissues. This process is thanks to the activation of host cell signaling after a recognition process resulting in the activation of P/MTI or ETI [6].

To enter into the plant, the microbes need to break off the cell barriers. To penetrate the cuticle and the cell walls, microbes produce cell wall-degrading enzymes such as cutinase, pectinase, cellulase, hemicellulase, protease and lignin-peroxidases [79]. As an example, the proteinase was abundant within fungal membrane vesicles and in the plant and/or fungal cell walls at the time of infection while absent in fungal pure culture [79]. To colonize the host tissues, endophytes needs to interfere with the defense signaling, for example *Piriformospora indica* interferes with the host cell death program to form a mutualistic symbiosis with plants [80]. The rhizospheric bacteria may enter and establish as root endophytes through emergence of lateral roots or root hair cells, primary and lateral root cracks, and diverse tissue wounds occurring as a result of plant growth [58].

Both abiotic factors and host genotype interact to control microbial communities. These variations act directly on a small number of closely linked taxa and exert a strong effect on communities, via microbe–microbe interactions, transmission of the effects to the microbial

community, and causing changes of plant microbial community structures [81]. Moreover, the microbial metabolic pathways of colonization may play an important role as determinants of endophyte diversity. For example, the rate of motile bacteria isolated from the interior part of roots was approximately fivefold higher than that of bacteria in the soil tightly adhering to the roots [82]. It has been proved that the ability of soil bacteria to approach plant roots is induced by chemotaxis and the efficiency in microcolony formation. These are the key factors that determine the success of bacteria to become endophytic [82]. Metagenomic analyses of bacterial microbiota in plants have shown that the phylogenetic and taxonomic composition of such microbial communities is limited to few bacterial phyla, including actinobacteria. Endophytic bacteria show a tremendous diversity not only in plant hosts, but also in bacterial taxa [36].

III. Effect of endophytes on plants growth promotion

Endophytes can enhance plant growth through multiple processes; actually two of them are largely harvested:

(i) Improvement of the plant nutrition through providing essential nutrients inaccessible or in a poor concentration into the soil. As an example microbes thanks to the action of specific group of enzymes, can solubilize phosphate and potassium trapped in the soil into chemical complexes. As an example, *Pseudomonas sp.* can mediate phosphate solubilizing in rice and wheat by producing gibberellic acid. In the addition, certain endophyte can convert atmospheric nitrogen (N_2) into organic form (NH_3^+) used by the plants. Certain microbes can provide micronutrient as the iron by the production of specific proteins like siderophores that bind iron. Finally endophytes have the ability to decompose organic components, including lignin, cellulose and hemicelluloses, which facilitate in nutrient cycling [83].

(ii) The second process for plant growth stimulation is associated to the ability of endophytes to modulate hormone concentration by excretion of certain growth or stress hormones as auxine, cytokinine, gibbereline and abscisic acid. Microbes can also manipulate the concentration of hormone into the plants by degrading their precursors, for example several strain produce ACC (Acide 1-aminocyclopropane-1-carboxylique) desaminase enzyme which is able to degrade the ethylene precursor, the ACC [83].

IV. Plant protection and bio-control effect of endophytes

IV. 1. Endophyte effect protection against stresses

Endophytes ameliorate plant responses to stress by multiple ways, as biosynthesis of antistress biochemicals by endophytes [75]. These microbes can also stimulate plants immunity and enhance the plant tolerance to the stress through activation of specific signaling pathways. Certain MAMPs like the flg22 peptides [84] and ß-glucan [85] that are found in respectively the bacterial flagella and the fungal cell wall can trigger MTI. Those conduct to the increase of the immunity level and thus reduce the impact of biotic and abiotic stress on infected plants [44]. Moreover, endophytes can also induce the overproduction of antimicrobial or immunological compounds by plants. As an example, *Trichoderma hamatum UoM 13* can induce the overproduction of endogenous SA and the overexpression of defense enzymes and Pathogenesis Related (PR) protein [86]. Endophytes ensure symptomless survival in plant tissues in two ways: one is that the microbes produce toxic metabolites to counter those of the host and mediate a host endogenous defense response by influencing phytohormone concentrations; the second way is that plants detoxify constitutive defense metabolites and secrete lytic enzymes. Endophytes participate in the regulation of redox stat of inoculated plants, by production of ROS scavengers including glutathione, ascorbate and tocopherol, and the enzymes, superoxide dismutases (SOD), catalases (CAT), ascorbate or thiol-dependent peroxidases (APX), glutathione reductases (GR), dehydroascorbate reductases (DHAR) and mono-dehydroascorbate reductases (MDHAR, [79]).

Endophytes play a great role in the amelioration of plant adaptation to abiotic stresses (drought, salinity, pollutes…etc.). Endophyte-associated plants (panic grass, rice, tomato and dune grass) have been reported to use significantly less water, increased biomass than in non-symbiotic plants. These microbes enhance resistance to drought by the reduction of leaf conductance and a slowdown of the transpiration stream [79]. Endophyte manipulates secondary signals as phytohormones (e.g. ABA, ethylene), ROS and intracellular second messengers (e.g. phospholipids) to enhance plant resistance. Root-derived ABA secreted by endophytes can ascend with transpiration flow to regulate stomata aperture in leaves under drought. The ability of grass species *Dichanthelium lanuginosum* to survive in soil temperatures ranging between 38 and 65°C in Yellowstone National Park was directly linked to an association with the fungus *Curvularia protuberata* and its mycovirus [87]. Another good example of amelioration of plants stress responses by endophytes is the inoculation of

the barley by *P. indica* that‟s induces salt tolerance by increasing the levels of antioxidants [88].

IV. 2. Endophyte effect protection against pathogens

Another important process of plant protection by endophyte against invaders is antagonisms occurring by production of antimicrobial compounds by endophyte against pathogens [30]. An example of this mechanism is *Paraconiothyrium SSM001*, which acts as a fungicide against host pathogens by producing taxol, which suppresses the mitosis of other fungi. *In vitro*, co-culture of *SSM001* with other fungi from its host plant (*Alternaria* or both *Alternaria* and *Phomopsis*) can stimulate taxol biosynthesis via *SSM001*, either directly or via their metabolite. In plants, pathogens and their diffusible chemicals, such as chloromethane and chitin, can induce the expression of genes related to the taxol biosynthesis and the release of taxol by *SSM001*. Yew treen can interact with *SSM001*, during pathogens attack, since Yew treen perceives pathogen entry or branch cracks, *SSM001* can migrate to the perceived points where taxol is specifically released. Actually, antagonistic endophytes were shown to be mainly fungal endophytes, and they were found primarily in weed and medicinal plant samples [89].

IV. 3. Endophyte effect protection against herbivores

Endophytes participate into plants responses against herbivore by production of toxic compounds against and thus protect plants from animal ingestion. For example several fungi can produce bioactive alkaloids, while root-colonizing pseudomonads, may directly act against plant-feeding insects by producing Volatile Organic Compounds (VOCs) that have insecticidal properties [36].

V. Bioremediation by endophytic microbes

In addition to describe effects, endophytes displays a various range of other benefits applied. Endophytes can degrade several pollutes *in* or *ex planta*, that‟s include heavy metals, hydrocarbons, volatiles compounds (carbon dioxide, methane, nitrous oxide, and halogenated compounds) and other contaminant as phenols, toxic dyes, pesticides…etc. A promising area of exploitation of endophytic bacteria for phytoremediation of contaminated environments has been described. Large numbers of bacterial strains isolated from grapevine (*Vitis vinifera L.*) plants were resistant to lead, mercury, nickel, zinc and manganese [90].

Biodegradation of pollutes is associated generally with microbial growth and metabolism; microbes catabolize pollutes and used it for their growth. In phytoremediation processes, selected or engineered microorganisms have been recently used in order to enhance phytoremediation. Numerous studies have demonstrated that endophytic microorganisms can accelerate these processes efficiently by interacting closely with their host plants[91]. These microorganisms reside inside both specific plant tissues and the root cortex or the xylem. Endophytes can also colonize dead and hollow hyaline cells of the plant genus *Sphagnum* which can be used for assimilation of methane, fixation of nitrogen, bioremediation of pollutants (e.g., pesticides, herbicides, insecticides, petrochemicals, polychlorobiphenyls, phenols/chlorophenols) and biotransformation of organic substances such as the propylene converting to epoxypropane and chiral alcohols. The methanotrophic endophytes inhabiting *Sphagnum spp*. can act as natural methane filter that can reduce CH_4 and CO_2 emission from peat lands by up to 50 % [82].

Several studies related to heavy metal decontamination (direct or indirect) by endophytes have been published. As example the bacterium *Bacillus sp.* reduced cadmium to approximately 94 % in the presence of industrially used metabolic inhibitors N,N′-dicyclohexylcarbodiimide (specific ATPase inhibitor, DCC) or 2,4-dinitrophenol (DNP). Similarly, inoculation with endophytic bacteria, *Serratia nematodiphila LRE07*, alleviated growth inhibition in *Solanum nigrum L*. in the presence of cadmium [92]. Endophyte can enhance plant growth in the presence of heavy metal by manipulation of the hormones, alteration in the levels of 1-aminocyclopropane- 1-carboxylate (ACC) by *Pseudomonas* and *Gigaspora* can alter the tolerance of heavy metals directly through the manipulation of plant ethylene levels [79].

Interestingly, recombinant endophytic bacteria are easier in application than genetic plants because their strains can successfully colonize multiple plants. Underling the importance of these microbes application in the area of bioremediation.

Introduction to the problematic of the study

M. truncatula is one of the most studied plants in the world, especially for the deciphering the legume-rhizobium symbiosis. It was shown that‟s legume nodule, in the addition to the rhizobium, can host also non-rhizobium species [93]. Actually little is known about Medicago relationship with endophytes and how the endophytes could potentially penetrate the nodule.

This project aims to launch the first investigations for understanding the tripartite relationship between endophytic bacteria-*Medicago*-*Sinorhizobium*. For that the followed steps were realized:

1- Creation of endophytic bacteria strain library for *Medicago*.

2- Validation of the endophytic behaviors of the isolated colonies.

3- Selection and characterization of selected endophytic bacteria

4- Study of the impact of selected strain on Medicago stress tolerance

5- Evaluation of Medicago immune response to the endophytic bacteria

6- Analysis of the endophytes on *Medicago*-*Sinorhizobium* symbiosis.

During this study five endophytic bacteria strain was analysed. The next sections describe the material and method employed during the study and the obtained results.

Part II. Materials and Methods

I. Plant materials, growth condition and stress treatment

I.1. *M. truncatula* preparation, culture and stress treatment

Seeds of *M. truncatula* ecotype A17 and R108 were first scarified with sulfuric acid treatment 7 min and washed 4 times with sterile water. Seeds were then with solution of sodium chloride at 5° for 20 min and washed abundantly using sterile water to eliminate traces of chlorine. Sterile seeds were stratified at 4°C for 48h in dark. Seeds were then germinated in growth chamber for 48h in the dark. Seedlings were transferred in buffered nodulation medium (BNM) supplemented with 50 mM of potassium nitrate (KNO_3^+) in 12×12cm plate containing eight seedlings. Plants were cultivated in a growth chamber at 26°C at 60% humidity with a photoperiod of 16h/8h of light/dark respectively [94]. For the salt stress test, inoculated and non-inoculated seedlings were transferred in BNM plate containing or note 100µM of NaCl [95]. The followed equation was used for the determination of the growth inhibition:

(Roots length control-roots length NaCl/ Roots length control + roots length NaCl) X 100

I.2. Bacterial culture and plant inoculation

Sinorhizobium medicae WSM419 [96]or endophytic bacterial strains (BT37, M50, M62.2, M17 and M18) are produced on yeast extract broth (YEB)-agar medium. After 48h of grown on agar plate at 30°C, bacteria are collected and suspended into sterile physiological serum. The inoculum is made by the dilution of the bacterial suspension and the adjustment of the optical densities to OD600=0.1. For the inoculation of Medicago: 1ml of the inoculum suspension was added per plat and the liquid excess was removed by pipetting.

II. Isolation of the endophytic bacteria

M. truncatula A17 3 day‟s old seedling were cultivated on mixture of compost and wild soils 1/1 v/v. The wild soils provide from the region of Deldoul (town of Ain El Ibel, city of Djelfa, Algeria) and organic farm located in Magtaa el Ouest (town of Taadmit, city Djelfa . The plants were cultivated in fields condition during the period starting form 15th of April 2021. After 40 days of growth, the roots were collected and their surface was sterilized in solution of chlorine 5°. After four wash with sterile water, the roots were crushed in solution of 50mM of Tris adjusted pH 6.5 with HCl. To remove debris, the lysate was centrifuged at

7000 rpm 5 min and the supernatant was collected and spread on ISP2-agar medium. The colonies formed were then collected, produced on IPS2-agar.

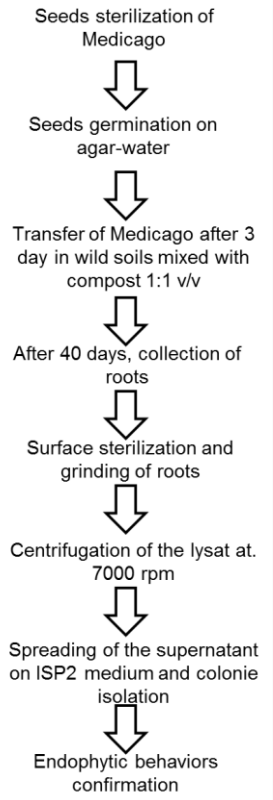

Figure 1. Method employer for the creation of Medicago-endophytic strain library. After sterilization of the seeds, the germination was done on agar-water plats. The 3 days germinated plants were transferred in soils mixed with compost and the plants were grown in natural condition. The roots of 40-days old plants were collected, surface sterilized and grind. To avoid debris, the lysats were then centrifuged at 7000 rpm and the suprenatantes are collected and spreaded in IPS2 rich growh medium. Finaly the obtained colonies were purified and a confirmation of their endophytic status is realized by inoculation of Medicago plants in axenic conidition.

III. Colonies Forming Unite (CFU) analysis

To determined CFU, roots of 14 dpi Medicago plants are collected. Roots surface is sterilized by incubation 5 min under checking in chlorine solution at 5° supplemented with 0.02% SDS. The roots are then washed four times using sterile water and the organ are crushed in sterile physiological solution. The suspension is spotted in YEB agar plate, three days after incubation in 30°C, the number of the colonies were determined.

IV. Acetylene Reduction Assay (ARA)

To nitrogenase activity is determined using the ARA. For that, plants were harvested after 21-dpi and individual plants were incubated with 500 µL of acetylene for 2h at room temperature in a 21 mL air tight glass vials sealed with rubber septa. After incubation, 200 µL of gas samples was removed from the vial and was injected into a gas chromatography system (7820A; Agilent Technology) to determine the ethylene production [97].

V. RT-qPCR expression analysis

RNA extraction, cDNA synthesis and RT-qPCR were per- formed as previously described [98]. After freezing in liquid nitrogen, the roots collected from 4 plants per experiment were ground in a 2 mL tube with beads and the total RNA was extracted using a TRI Reagent procedure recommended by the manufacturer (Molecular Research Center). DNA was removed from the samples using the DNAse I kit (Invitrogen) as recommended by the manufac- turer.

The concentration and the RNA quality were checked using the NanoDrop ND-1000 (Thermo Scientific). Reverse transcription was performed on 1 µg of the total RNA (DNA free) using oligo dT and SuperScript II (Life Technology) according to the supplier in a final volume of 20 mL.

For each tested gene, the primers amplified 200–300 nucleotides of the cDNA sequence and the quantification was made using qPCR on a LightCycler 480 (Roche Life Science) with the LightCycler FastStart DNA Master SYBR green I kit according to manufacturer‟s instructions (Roche). The temperatures of 94C°, 58–62C°Cand 72C° were used, respectively, for denaturation, annealing, and extension steps. In all analyzed samples, expression levels were normal- ized using the housekeeping gene MtACT (Actin 11, [99]).

VI. Molecular strain identification

16S rRNAs were amplified by PCR using the PA and PH primers on colonies sampled from a 16 hours on YEB-agar plat. A colony is re-suspended on 20 µl of sterile water and 4 cycles of freezing liquid nitrogen and boiling at 48°C were realized. 1 µl of cell lysate was amplified using Q5 High-Fidelity DNA Polymerase® following the instruction of the manufacturer. The PCR product was then purified using MinElute PCR Purification Kit® of Qiagen. Purified products were then sequenced by Eurofins (https://www.eurofins.fr/) and the obtained sequences were submitted to NCBI GeneBank for the identification of homologous sequences and the construction of the phylogenetic tree.

VII. Phylogenetic tree construction

The evolutionary history was inferred using the Neighbor-Joining method [100]. The optimal tree with the sum of branch length = 0.73575388 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are

shown next to the branches [101]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [102] and are in the units of the number of base substitutions per site. This analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1717 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [103].

VIII. Rhizobia- endophyte co-inoculation experiment

S. medicae strains WSM419 [104]expressing *LacZ* provided by G. Endre [105] and wild type endophytic bacteria BT37, M17, M18, M50 and M67.2 were used. The bacteria were cultivated in liquid YEB medium for 24-48h at 30°C supplemented with tetracycline at 5 µg.mL-1 for the LacZ strain. Inoculation suspension is prepared by centrifugation of the bacterial culture at 14 000 rpm 5 min. The supernatant were removed and the bacteria are re-suspended in sterile water. To wash the bacterial cells, the process is repeated. The inoculum is made by the dilution of the bacterial suspension and the adjustment of the optical densities to OD600=0.1. The plant were inoculated with *S. medicae WSM419* alone or with mixture of *S. medicae WSM419* and endophytic bacteria at 1/1 V/V. 14-dpi nodule were collected, surface sterilized using chlorine 5° solution supplemented with 0.02% SDS. The nodule are then washed four times using sterile water and the organ are crushed in sterile 50 mM Tris-HCl solution with pH= 7. The suspension is spotted on YEB agar plat supplemented with X-gal, 50µg/ml and IPTG 100µg/ml.

IX. Enzymatic activities determination

IX.1. Pectinase production

Pectinase enzymes were tested in the ASO medium with 1% pectin added. Petri dishes seeded by spot by the bacterial strain are incubated at 28.2°C for 10 days. After adding 10mL of Lugol solution to the cans, the appearance of a clear halo around the colonies indicates a positive reaction [106].

IX.2. Cellulase production

Transplant of isolates obtained is carried out on a medium added to CarboxyMethyl Cellulose (CMC) according to the method of Lu et al 2004. Petrie dish are seeded by spots, then incubated for 10 days at 28.2°C. After incubation, a solution of Congo red (1%) is added to the surface of the colonies for 15min, then after removal of its excess from the surface, once again the surface of the petri dishes is flooded with NaCl (1M) for 15 min. The appearance of a clear halo around the colonies reflects the presence of cellulase [107].

IX.3. Protease production

Qualitative evidence of proteolytic activity consists in seeding bacterial isolated on the skim milk agar medium. After incubation for 10 days at 28.2°C, protease production is indicated by the presence of a colorless area around the culture on the same culture medium [108].

IX.4. Lipase production

Lipase activity was demonstrated on selective media containing tween 20, 40 and 80. After transplanting isolates to media and incubating at 28.2°C for 10 days, lipase activity was determined based on the presence of deposits around colonies [109].

Part III. Results

I. Creation of *M. truncatula* roots endophytic bacteria collection

In the aim to develop a strain library of endophytic bacteria with possible PGPR effect and/or nodule colonization ability. Isolation of potential endophytic bacteria was realized with the protocol described in the material and methods. *M. truncatula* R108 and A17 plants were grown in a mixture of compost and wild soils. The soils were collected from desert areas in Daldoul, Ain El Ibel (Algeria) or in organic farm in Magtaa el Ouest, Taadmit (Algeria). This experiment leads to the isolation of 80 potentials endophytic bacteria.

In the aim to validate the endophytic behavior of the isolated bacteria, analysis of the roots colonizing ability of the strain was realized for 46 strains by inoculation of *M. truncatula* A17 in axenic condition. For this purpose the plants were inoculated with the potential endophyte and evaluation of the roots colonization by the bacteria was realized by CFU analysis of 14 days post inoculation (dpi) roots. The results of the Figure 2 display the number of plants that show no (CFU<10), medium (10<CFU<100) and high (CFU>100) tissues colonization.

Based on the number of plant displaying CFU superior than 100 (high colonization), five groups can be distinguished with different level of roots infection by the endophyte (Figure 2B):

The group 1 correspond to plant that show very low level of roots colonization by the endophytes; plant show mainly CFU less than 100.

The group 2 displays plants with low level of roots colonization: no more than one plants show CFU up to 100.

The group 3 and 4 represent plants displaying high level of tissues colonization: between 2 and 3 plants shows CFU superior to 100.

The groups 5 encompass plants with high roots infection by the endophyte: more than 4 plants display CFU up to 100.

Together these results confirm the endophytic status of isolated bacteria and indicate that tested strain are in majority (40/46 strains) able to colonize efficiently the roots, with different level of infection.

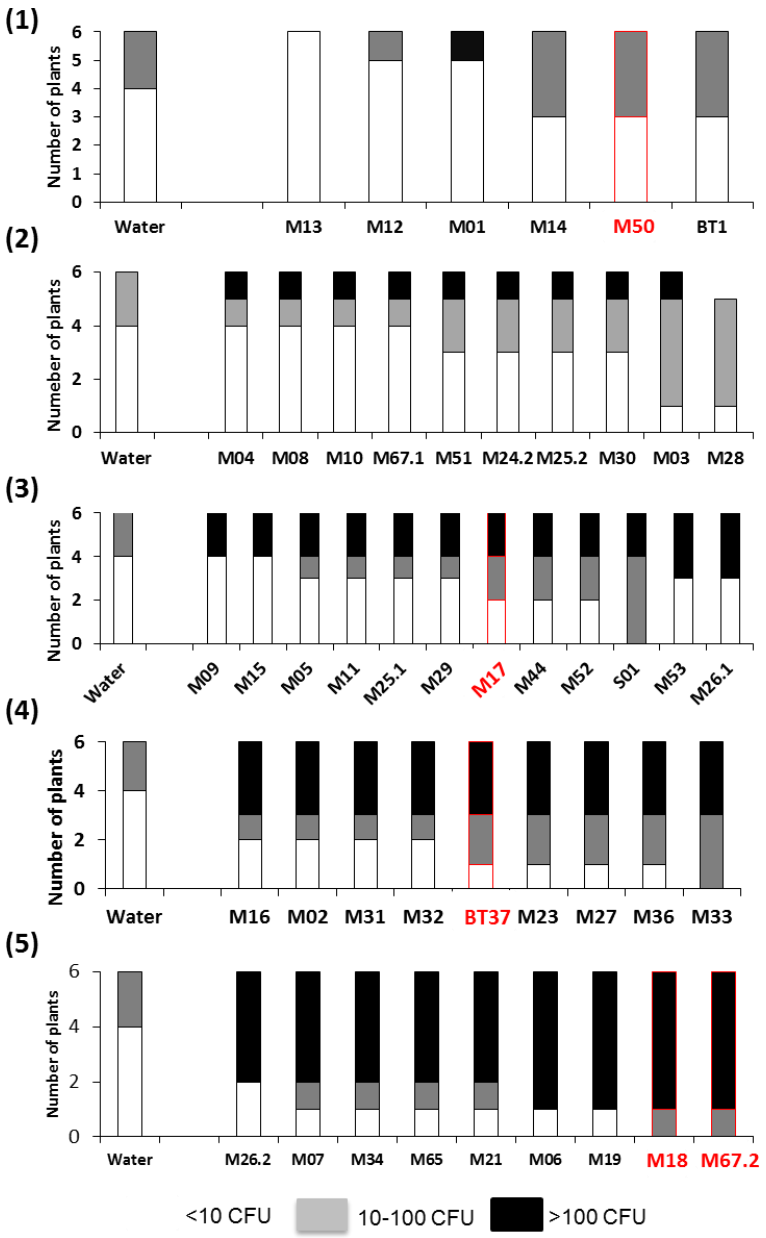

Figure 2. Endophytes colonize the roots of Medicago with different rates. The endophytic bacteria display different level of roots colonization. Based on the number of plants displaying CFU superior to 100 (black color), five groups are distinguished: group 1 the strain with low level of colonization, the group 2 to 4 display endophyte with intermediate colonization level and the group 5 correspond to the highly infected plants. In white the number of plant showing CFU<10, in grey the number of plants with CFU between 10 to100 and in black the number of plants with CFU more than 100. The results were obtained from 14-dpi *M. truncatula* A17 plant inoculated with endophytes. Two independent experiments with 3 biological replicates were used. In red the selected strain for the characterization.

II. Diverse bacterial species can colonize Medicago roots

Five strains (M50, M17, BT37, M18 and M67.2, Figure 3A red color) showing different level of colonization were randomly selected for the characterization steps. The observation of the bacterial colonies (Figure 3A) reveal similar morphotype between all strains, which producing mucus, except for the strain BT-37 that seem don‟t produce mucus (Figure 3C).

The identification of the strain species was realized by sequencing the16S rRNA gene. For that, 1.5 kb fragment of 16S rRNA of all the isolates was amplified by PCR. After purification the PCR products were sequenced using PA forward primer. Nucleotide sequences of the isolates were compared with sequences available in NCBI GenBank. The percentage of 16S rRNA gene sequence similarities (70–99.8 %) were selected for the identification.

The 16S rRNA sequencing were successfully done for the strains M17, M18, M67.2 and M50 and reveal that these bacteria are belonging to Pseudomonadaceae (M17), Bacillaceae (M67.2 and M50) and Enterobacteriaceae (M18, Figure 3B). The first hit of the blast on NCBI Gene Bank reveal that M17 sequence correspond to *P. aeruginosa PaLO34*, M18 show high similarity with *Enterobacter sp. U4*, M50 correspond to *Lysinibacillus sp. 7B* and M67.2 correspond to *B. amyloliquefaciens W36* (Table S1). The quality of the sequence for the16S rRNA of the strain BT-37 was pretty low. The preliminary analysis indicates that this strain is potentially belonging to *Streptococcus sp*, BT-37 16S rRNA show 49%/74.50% of coverage/identity with *S. pseudopneumoniae HMCB45* (Table S1, Figure 3.D).

Together these results indicate that diverse bacterial genera and species were isolated and suggest that a wide range of bacteria are able to colonize the *M. truncatula* roots tissues.

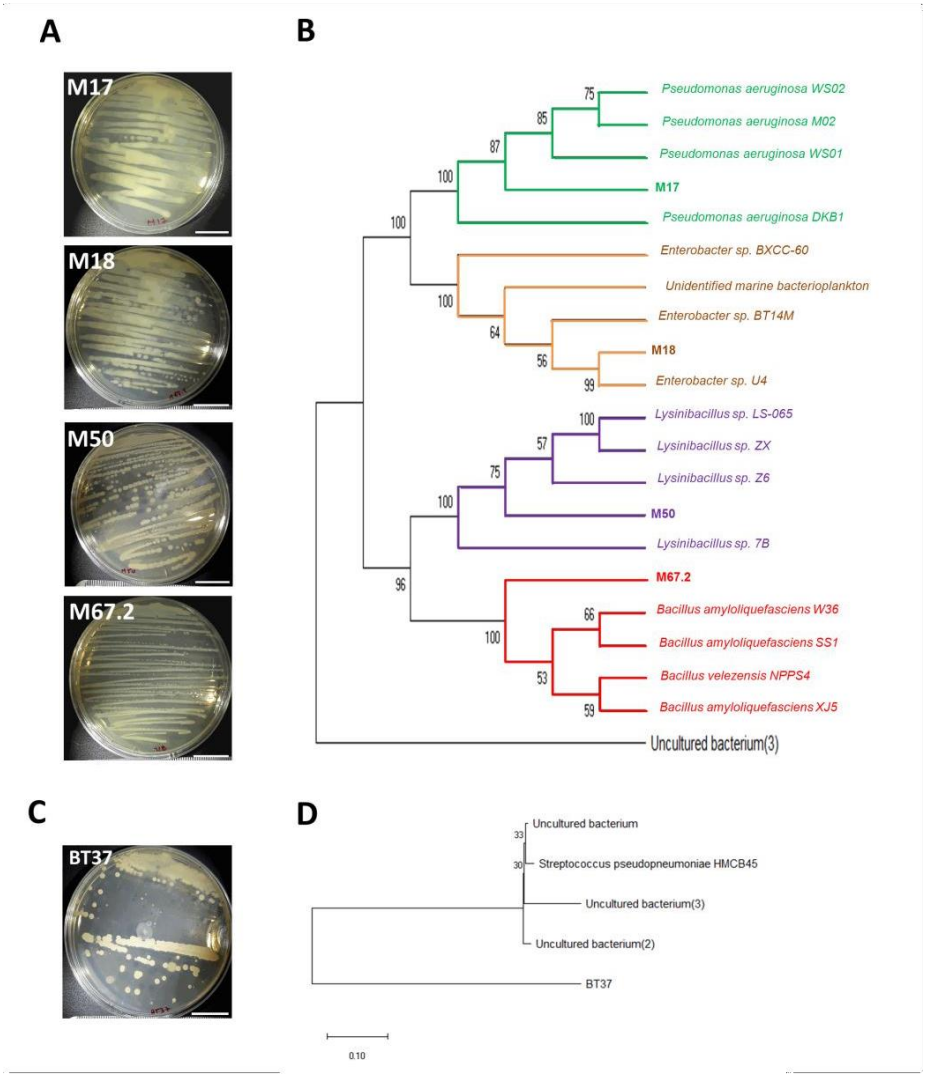

Figure 3. Identification of bacterial strain isolated from roots of Medicago. (A) Colonies morphotype for the strains M17, M18, M50 and M67.2, scale bar is 2 cm. (B) Phylogenetic tree based on rARN 16s gene sequence showing position of the endophytes to taxonomically similar bacteria for M17, M18, M50 and M67.2. 16S rRNA sequence of uncultured bacterium was used as out group for the tree rooting (C) Colonies morphotype for the strains BT-37, scale bar is 2 cm. (D) Phylogenetic trees based on rARN 16s gene sequence showing the putative position of BT-37 to taxonomically similar bacteria.

III. Studied endophytic bacteria infect potentially the *M. truncatula* roots trough different way

To determine the ability of endophyte to infect the *Medicago* R108 ecotype. The analysis of the roots infection was determined at seven and forty days after the plants inoculation. Analysis of CFU of inoculated plants reveals that at 7 dpi all the bacteria are detected into the roots, suggesting an early interaction between endophyte and Medicago. Comparison of strains infection rate reveal that like in A17, *Lysinibacillus sp* (strain M50) show the lower ability of roots colonization in R108 (Figure 4A Vs. Figure 2). By contrast to A17, in R108, *Streptococcus sp.* (BT-37) is the strain with the higher infection rates. The others strain shows also high ability of roots tissues occupation of R108 (Figure 4A) as it‟s observed in A17 (Figure 2).

To investigate the potential bacterial mechanism involved during the roots invasion, key enzymatic activities (pectinase, lipase, protease and cellulase) related to endophyte plant tissues colonization were determined (Figure 4B). *Streptococcus sp.* is most active strain with three from the four tested activities: pectinase, lipase and cellulase. *P. aeruginosa* display pectinase and cellulase activites. *Enterobacter sp.* and *Lysinibacillus sp.* show only lipase activity, whereas *B. amyloliquefaciens* don‟t process any of tested activities. These observations indicate variable enzymatic activities between the tested strains and suggest that various processes could be used by endophyte to colonize Medicago roots.

IV. Co-occurrence is observed between the infection rate and the stimulation of Medicago roots immunity

To investigate the plant immune response to the endophytes, analysis of defense markers genes *PR10, PR5* and *NDR1-Like* was assessed in 14-dpi roots of *Medicago* A17 plants. The Figure 4C shows the obtained results. In all tested strain, the defense markers are up-regulated. Interestingly, the expression level of *PR10* (Figure 4C) is linked with the infection rate of A17 roots by the endophytes (Figure 2): *Lysinibaccillus sp.* (M50) show the lowest *PR10* induction and *Enterobacter sp.* (M67.2) display the most important induction folds. The others strains displays intermediate induction folds (Figure 4C).

By contrast to the *PR10*, the expression analysis of the other genes (*PR5* and *NDR1-Like*) indicate different level of stimulation depending to the strain: *Lysinibaccillus sp.* has the lowest induction folds, by contrast *P. aeruginosa* and *Enterobacter sp.* show the highest

induction of *PR5* and *NDR1-Like*. To gather these results indicate that the immunity stimulation is linked to the infection rates of the endophyte.

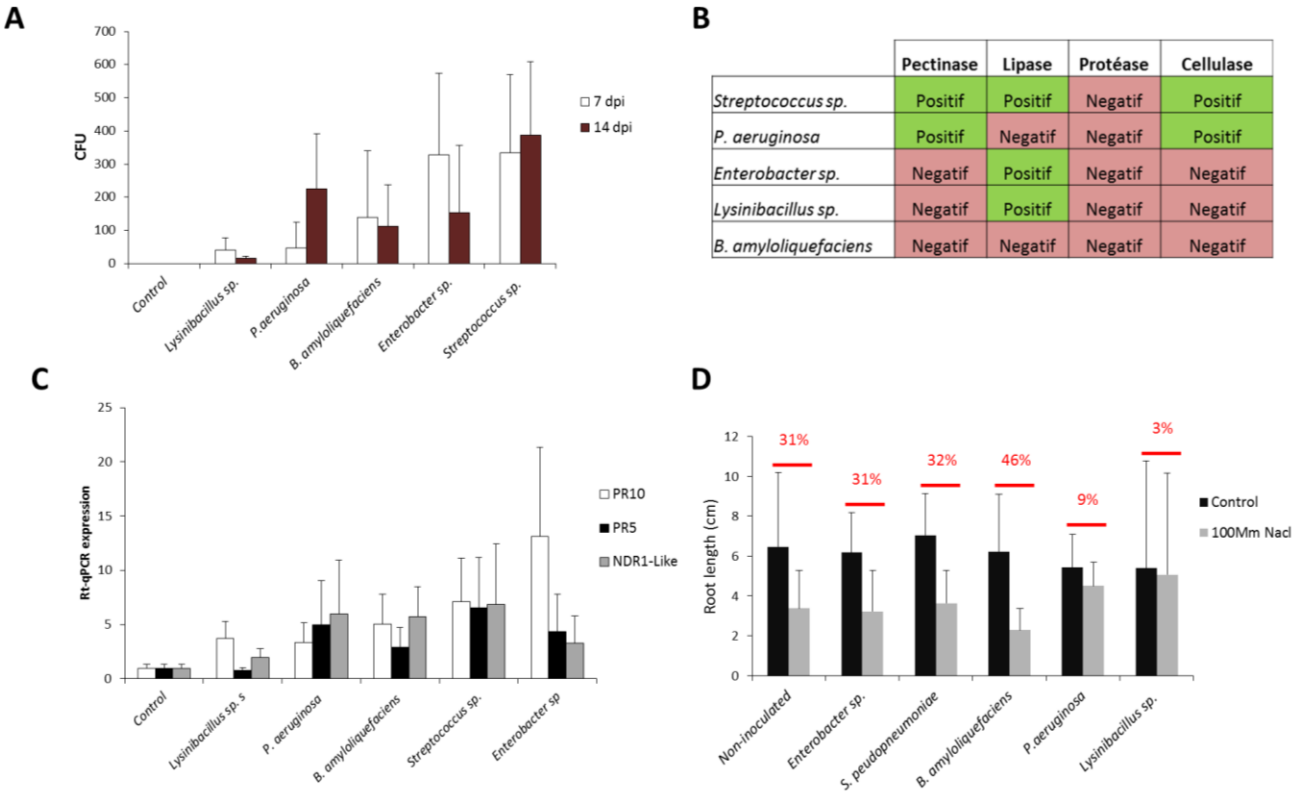

Figure 3. Medicago-endophyte interaction. (A) CFU analysis of *M. truncatula* R108 plants at 7- and 14-dpi with endophytes. The graphic show the results of one biological repetition with three technical replicates. The error bare shows the Standard Error (SE). (B) Summery of obtained results for pectinase, lipase, protease and cellulase activites for the studied endophytes. (C) RT-qPCR analyses of *PR10*, *PR5* and *NDR1-Like* defense genes in 14-dpi *M. truncatula A17* roots. The expression is normalized using the actine house keeping genes. The graphic show the mean expression obtained from two biological repetitions and two technical replicate. The error bare show SE (D) Root growth analysis of *M. truncatula* R108 plants cultivated in presence or absence of 100mM NaCl. The red value displays the percentage of roots growth inhibition by the NaCl. The results show the mean growth of plants from two biological repetitions, with at least three plants. The error bare show SE

V. Endophyte infection ability is in opposite with the Medicago protection from the salt stress

To evaluate the potential protection against abiotic stress of Medicago by the endophytes. Analysis of roots growth inhibition during salt stress was investigated by treating or not plants with 100 mM NaCl in absence or in presence of the endophytes (Figure 3D). The salts reduce roots growth with around 31 to 32 % in the control or in presence of *Enterobacter sp.* or *Streptococcus sp* (Figure 3D). The inhibition is enhanced in plants inoculated with *B. amyloliquefaciens* with 46% of growth inhibition. By contrast, the salt effects were reduced in plants inoculated with *Lysinibaccillus sp.* and *P. aeruginosa* which shows 9% and 3% of growth inhibition respectively.

Interestingly the comparison of roots infection rate of endophytes (Figure 2, Figure 3A) and salt stress protection (Figure 3D) indicate the presence of opposition between infection ability and the protection conferred by the strain against salt stress: *Lysinibaccillus sp.* and *P. aeruginosa* which displays the lowers infection ability, gives the better plant protection. By opposite, *B. amyloliquefaciens* which display the most efficient roots colonization aggravate the roots growth inhibition in response to the salt.

VI. Endophytes display low impact on early SNF interaction and affect nodule physiology

To investigate the interactions between endophytic bacteria and rhizobia during the nitrogen-fixing symbiosis (SNF). The analysis of the impact of endophyte on nodulated plants were analyzed. To discriminate between endophytic bacteria and the endosymbionte of *Medicago*, the bacteria *S. medicae WSM419*, a modified transgenic strain of *WSM419* was used, the *LacZ* expressing strain (Figure 4A). The *LacZ WSM419* bacteria produce the LacZ enzyme, which is able to degrade colorless X-Gal substrate on blue product [110]. The blue color of the *LacZ* expressing strain was used to discriminate between endophytic bacteria and S. *medicae WSM419*.

To determine the time point for the response analyses, a kinetic of nodulation was firstly realized (Figure 4B). *M. truncatula* A17 plant were inoculated with the *WSM419* strain and the nitrogen fixation were analyzed from 1 to 13-dpi plant cultivated *in vitro*. In our condition of culture, nitrogen fixation start at 9-dpi and increase upon the infection time. 17-dpi was selected as time point of the analysis of endophyte-rhizobium interaction.

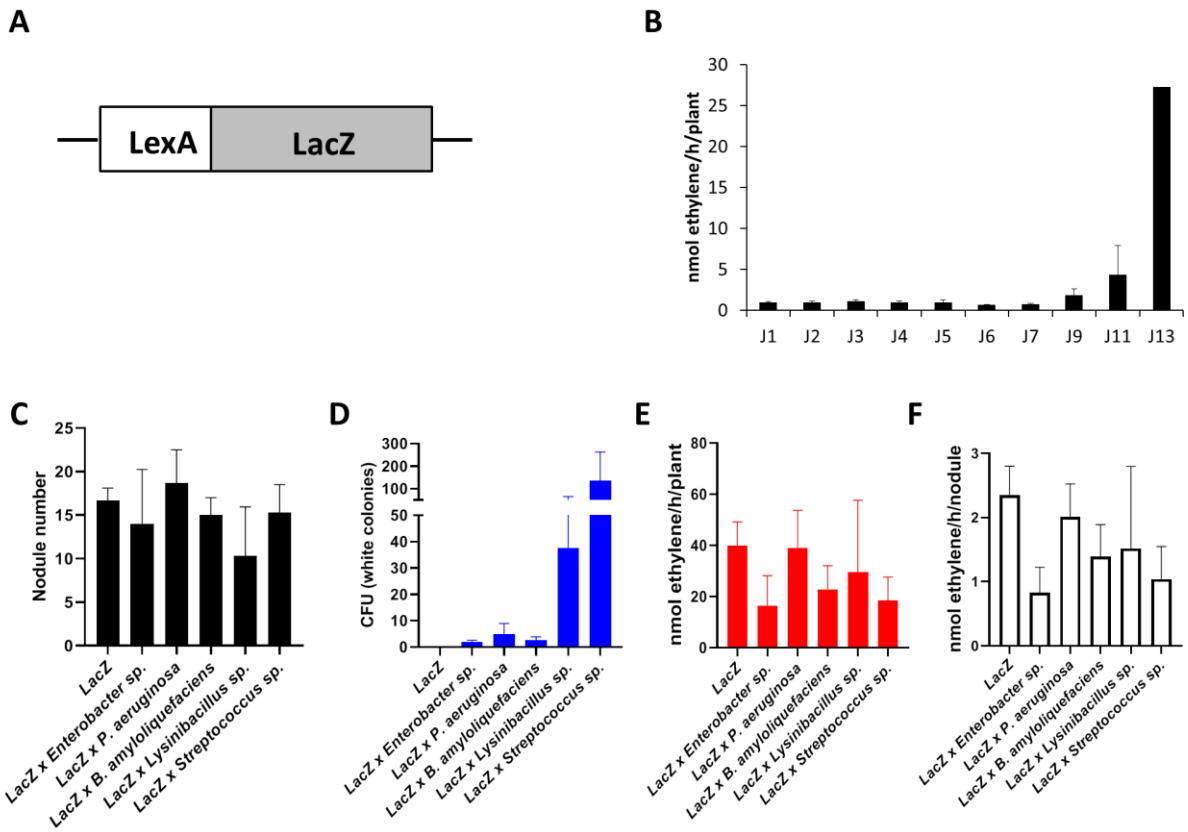

Figure 4. Endophyte-Rhizobium-Medicago interaction. (A) Structure of the transgene used in *S. medicae WSM419* to discriminate between the rhizobim and the endophyte during the experiments. The *LacZ* gene is fused to the constitutive promoter LexA. *LacZ* confer blue color to *S. medicae WSM419* in presence of the X-Gal. (B) Analysis of nitrogen fixation ability of *M. truncatula* A17 plants during kinetic of nodulation with *S. medicae 419*. Three biological experiments with at least 8 plants were used. The error bare shows the SE. J represent the DPI. (C) Number of counted nodules of *M. truncatula* A17 plants inoculated with *S. medicae 419 LacZ* alone or co-inoculated with endophytes. (D) CFU analysis of white bacteria isolated from nodules of *M. truncatula* A17produced by *S. medicae 419 LacZ* alone or co-inoculated with endophytes. The CFU were determined in YEB growth medium containing X-Gal. (E) Nitrogen fixation analysis of *M. truncatula* A17 nodulated plants with *S. medicae 419 LacZ* alone or co-inoculated with endophytes. (F) Nitrogen fixation analysis reported to nodules produced by *M. truncatula* A17 plants inoculated with *S. medicae 419 LacZ* alone or co-inoculated with endophytes. For C to F experiments two independent experiments with eight plants were analyzed. Error bare show the SE.

M. truncatula A17 plants were then inoculated with *S. medicae WS419 LacZ* alone or co-inoculated with endophytic strains. The analysis of the nodule number between WSM419 LacZ alone and co-inoculated plants reveal slight reduction of the nodule induced by *S. medicae*, suggesting a low impact of the endophytic bacteria on the early interaction (nodule formation) between *Medicago-S. medicae WSM419*.

To assess the ability of roots-endophytic bacteria to colonize the nodule. 17-dpi nodules were collected form plants inoculated with *LacZ* strain alone or co-inoculated with endophytic bacteria. The nodules were then surface sterilized and grinds. The obtained samples were spotted on YEB agar growth media containing X-Gal. The number of white colonies was then determined (Figure 4D). In all cases white colonies were identified suggesting that all tested endophytes are able to infect the nodule. *Lysinibaccillus sp.* and *Streptococcus sp.* show the most important CFU values, indicating that these bacteria are able to infect efficiently the symbiotic organ.

To analyze the impact of nodule colonization by endophyte on the nitrogen-fixation ability, ARA analysis was realized on nodulated plants in absence or in presence of endophytes. Nitrogen fixation is reduced in the presence of *Enterobacter sp.*, *B. amyloliquefaciens* and *Streptococcus sp* compared to non-inoculated plants. In response to *Lysinibaccillus sp.* the nitrogen fixation is slightly reduced compared to the control, whereas *P. aeruginosa* don‟t alter the nitrogen fixation ability (Figure 4E). The analysis of nitrogen fixation reported to the nodule number (Figure 4F) confirm the results of the nitrogen fixation analysis for the whole plants, suggesting a negative effect of the presence of most of the tested endophytes on the nodule nitrogen-fixation ability.

Together these results suggest that roots-endophytes slightly affect the early symbiotic interaction between *Medicago-S. medicae WSM419*, but they can colonize and affect the nodule functioning. Moreover, the effect on the nodule seems to not correlate with endophyte nodule colonization ability.

Discussion

This study aims to investigate *Medicago* relationship with endophytic bacteria and the impact of endophytes on *Medicago-Sinorhizobium* symbiosis. For that a collection of endophytic bacterial strain was generated by using soils sampled from desert or organic farms. It was shown that the exposition of plant to a prolonged stress enhance the attraction of beneficial microbes [111]. In the aim to maximize the recruitment of endophytic bacteria by *Medicago*, the plants were grown in natural (in fields) condition. This method of culture exposes the plants to a higher stress than *in vitro* or green house condition.

Eighty bacterial strains were isolated from the cultivated plants and forty six of them were checked for their ability to infect roots in axenic condition. Forty strains (86% of tested strains) display roots tissues invasion, indicating that's used method is efficient for fast development of endophyte strain library. Five strains (M17, M18, M50, M67.2 and BT-37) among the forty validated bacteria were selected for characterization. The molecular identification indicates that M17, M18, M50 and M67.2 are respectively related to *P. aeruginosa*, *Enterobacter sp.*, *Lysinibacillus sp.* and *B. amyloliquefaciens*. For BT-37, the sequence quality was pretty low and the preliminary data suggest that this strain is belonging to *Streptococcus sp*. However a re-sequencing is required for the validation of the BT-37 identification.

The genetic variations between the identified strains suggest that a wide range of bacteria are able to colonize Medicago roots tissues. The studied endophytes are able to infect the two tested *Medicago* ecotypes A17 and R108, indicating that the host genetic background display a low influence on the endophytes colonization of the host roots. Several differences are observed between A17 and R108, especially in the immune control during the symbiosis; R108 develop more defense response than A17 in response to symbiosis arrest [94,112,113]. Interestingly the levels of roots colonization by the endophytes change between A17 and R108, for example the strain *B. amyloliquefaciens* that"s show the higher infection level in A17, displays intermediate infection in R108. These observations could be explained by inability of certain endophyte to manipulate efficiently A17 and R108 immunity. According to this hypothesis, the analysis of enzymatic activities associated to endophyte infection reveals the presence of variability between tested strains. As an example *B. amyloliquefaciens* don"t show any of the tested activities, whereas *Streptococcus sp.* displays three among the four tested activities. These observations could be explained by different strategies engaged

by studied strain for the roots infection. Crack entry is among the strategy that's can potentially explaining the difference of roots infection by the endophytes. This process of infection consists on the penetration of the endophyte in cracks located between principal and secondary roots [58]. In addition to the crack entry, the bacteria can use type III and type IV secretion systems to manipulate host immunity during the infection [31].

Inoculation of A17 ecotype by studied endophytes leads to the up-regulation of *PR10, PR5* and *NDR1-Like* genes. Different level of immune stimulation is observed depending to the used strain. Interestingly, the *PR10* genes expressions correlate with the level of tissues occupation by the endophytes: *Lysinibacillus sp.* that display low tissues infections have smallest *PR10* up-regulation compared to the control, by opposite *B. amyloliquefaciens*, which is the most virulent strain display the higher *PR10* stimulation in comparison to non-inoculated plants. These observations suggest that the level of tissues occupation is linked to the defense stimulation in *Medicago*.

To investigate the potential PGPR effects of the studied strain, analysis of *Medicago* response to salt stress in presence or absence of endophytes was assessed. *Enterobacter sp.* and *Streptococcus sp.* display no difference with the controls, *B. amyloliquefaciens* aggravate the stress effect, by the opposite *Lysinibaccillus sp*. and *P. aeruginosa* reduce the impact of the stress on the roots growth. These results indicate the presence of various effects of tested strain on *Medicago* protection to abiotic stress. Interestingly *Lysinibaccillus sp*. and *P. aeruginosa* display the lowest infection rates in A17 and R108, suggesting that less infective strain can possess the higher protection to salt stress. According to that"s the *Enterobacter sp.* SA187 protect *Arabidopsis thaliana* from the NaCl stress and show epiphyte localization [114].

Finally to investigate the potential impact of endophyte on *Medicago-Sinorhizobium* symbiosis a co-inoculation of Medicago with the studied strain and the rhizobium was realized. Our preliminary data suggest that endophytes have a low impact on the initiation of the symbiotic interaction. This is represented by a small variation of the nodule number in the presence or in the absence of the endophytes. By contrast all tested endophytes are able to infect the nodules and reduce the nitrogen fixation ability. Interestingly, *Lysinibaccillus sp*. Tha's show the lowest ability for roots colonization, display the highest infection rates of the nodules. This observation underlie the difference between nodule and roots and could be explained by the difference of the immunity and/or of the metabolic status. Finally the

infection rates seems to don’t correlate with the nitrogen-fixation, certain strain such as *Streptococcus sp.* display high infection with high impact on nitrogen fixation, while other *Lysinibacillus sp.* have a smallest impact despite a higher infection.

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Supplemental information

Table S1. First hit identification

Strain	16S rRNA homologous	Max score	Total score	Query Cover	E. value	Per. Identity
BT-37	Streptococcus pseudopneumoniae strain HMCB45	228	228	49%	3.00E-54	74.50%
M17	Pseudomonas aeruginosa strain PaLo34	2298	9176	100%	0	99.60%
M18	Enterobacter sp. strain U4	1022	1022	90%	0	96.31%
M50	Lysinibacillus sp. strain 7B	2172	2172	99%	0	99.66%
M67.2	Bacillus amyloliquefaciens strain W36	2200	2200	100%	0	99.59%