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Development of a Genetic System in Plant Growth-Promoting Paraburkholderia

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“The value of an action depends on the intention behind it. A man will be rewarded only for what he intended”.

-Muhammad Rasulullah-

(Narrated by 'Umar bin Al-Khattab in Jami` at-Tirmidhi 1647, graded authentic)

I intended this to understand You.

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Abstract

This study explores the development of a genetic system in a novel plant growth promoting *Paraburkholderia* isolate belonging to the beta proteobacteria. The main objective is the investigation of the mechanisms through which this isolate promotes plant growth. Using an allelic exchange system, successful knockouts of the *nifH* and *acdS* genes were achieved. These knockouts were generated by amplifying upstream and downstream homologous flanks of the target genes, which were then integrated into a suicide vector (pMo130) for conjugative transfer into *Paraburkholderia* Msb3. This method allowed for precise gene deletions, confirmed via PCR and sequencing, enabling a deeper understanding of the roles and impacts of these genes on plant growth promotion.

The Msb3 strain demonstrated significant plant growth-promoting potential, primarily through the production of ACC deaminase, which reduces ethylene levels in plants—a hormone associated with growth inhibition under stress conditions. The ability of Msb3 to modulate ethylene levels by cleaving ACC (1-aminocyclopropane-1-carboxylate), a precursor to ethylene, suggests a key role in enhancing plant stress tolerance and promoting overall growth. In addition, although nifH knockout strains showed no impact on plant growth promotion under the experimental conditions, the presence of this nitrogenase gene cluster highlights the bacterium's potential for nitrogen fixation in natural environments. Together, these findings indicate that Msb3 employs multiple mechanisms—such as ACC deaminase activity and possibly nitrogen fixation—to enhance host fitness and promote plant growth, making it a promising candidate for sustainable agricultural applications.

Zusammenfassung

Diese Studie untersucht die Entwicklung eines genetischen Systems in einem neuartigen Paraburkholderia-Isolat, das zu den pflanzenwachstumsfördernden Beta-Proteobakterien gehört. Das Hauptziel besteht darin, die Mechanismen zu erforschen, durch die dieses Isolat das Pflanzenwachstum fördert. Mithilfe eines Allelaustauschsystems konnten gezielte Knockouts der nifH- und acdS-Gene erzeugt werden. Diese Knockouts wurden durch die Amplifikation homologer Flanken der Zielgene upstream und downstream generiert, die anschließend in einen Suizid-Vektor (pMo130) integriert und durch konjugativen Transfer in Paraburkholderia Msb3 übertragen wurden. Diese Methode ermöglichte präzise Gen-Deletionen, die mittels PCR und Sequenzierung bestätigt wurden, was ein tieferes Verständnis der Rolle und Auswirkungen dieser Gene auf die Förderung des Pflanzenwachstums ermöglichte.

Der Msb3-Stamm zeigte ein erhebliches Potenzial zur Förderung des Pflanzenwachstums, hauptsächlich durch die Produktion von ACC-Deaminase, die den Ethylengehalt in Pflanzen reduziert – ein Hormon, das unter Stressbedingungen mit Wachstumshemmung verbunden ist. Die Fähigkeit von Msb3, die Ethylenkonzentration durch das Spalten von ACC (1-Aminocyclopropan-1-Carboxylat), einem Vorläufer von Ethylen, zu modulieren, deutet auf eine Schlüsselrolle bei der Verbesserung der Stressresistenz von Pflanzen und der Förderung des Wachstums hin. Darüber hinaus zeigten nifH-Knockout-Stämme unter den experimentellen Bedingungen keinen Einfluss auf die Förderung des Pflanzenwachstums, jedoch weist die Anwesenheit dieses Stickstofffixierungs-Gens auf das Potenzial des Bakteriums zur Stickstofffixierung in natürlichen Umgebungen hin. Insgesamt zeigen diese Ergebnisse, dass Msb3 mehrere Mechanismen – wie die ACC-Deaminase-Aktivität und möglicherweise die Stickstofffixierung – einsetzt, um die Fitness des Wirts zu verbessern und das Pflanzenwachstum zu fördern, was es zu einem vielversprechenden Kandidaten für nachhaltige landwirtschaftliche Anwendungen macht.

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1. Introduction

The demand for food due to population growth and environmental issues is higher every day. This problem has led to a focus on increasing agricultural productivity through more sustainable and environmentally friendly approaches (Glick *et al*., 2007).

1.1. The beauty of plant microbiota

One of the many beauties that life sciences brought to humans is the knowledge that plants live in harmonious system with a diverse community of microorganisms, called plant microbiota. Research on the plant microbiota has revealed the complex interactions that exist between the plant, the associated microbial communities, and the environment, and has helped to understand how these interactions shape the composition of the plant microbiome and influence its beneficial traits. The plant microbiota plays a vital role in plant health and development, providing a range of benefits to the plant host including growth promotion, nutrient uptake, stress tolerance, and protection against pathogens (Trivedi *et al*., 2020).

1.2. Plant growth promoting bacteria

Among the diverse communities of plant microbiota lays another wonder of nature's design that works in concert with plants, creating a captivating and awe-inspiring web of life beneath the surface: the plant growth promoting bacteria (PGPB) (Glick *et al*., 2007). Research shows that PGPB can influence plant growth either indirectly or directly. Indirect influences include inducing systemic resistance so that a plant can respond to pathogen invasion more quickly and forcefully (Verhagen *et al*., 2004), or rapidly colonizing the plant tissue and consuming the majority of available nutrients, making pathogen growth difficult (Innenerbner *et al*., 2011). Direct plant growth influence can be achieved by helping a plant obtain resources that it needs for growth and development such as nitrogen (Islam *et al*., 2013). Another way in which PGPB can directly influence the plant growth is by adjusting the levels of phytohormones and they can do so by synthesizing the hormones themselves, or modifying the plant's ability to synthesize hormones (Spaepen *et al*., 2011; Glick, 2012). It is expected that PGPB will eventually replace the use of chemicals in agriculture and other fields, which makes it important for the strategies for promoting plant growth to be explored.

1.3. Habitats of plant growth promoting bacteria

These fascinating organisms have adapted to colonize and establish symbiotic relationships with plants in the distinct ecological niches of both rhizosphere, the soil region surrounding plant roots, and the phyllosphere, the aerial habitat encompassing the surfaces of plant leaves.

While extensive research has been dedicated to unraveling the structure of rhizosphere bacteriome (Ling *et al*., 2022) and intricate mechanisms by which PGPB exert their influence in the rhizosphere (Saeed *et al*., 2021), the study of phyllosphere-associated PGPB has received relatively little attention. This is despite the fact that bacteria stand out as the predominant and enduring inhabitants of the phyllosphere, exhibiting remarkable abundance and persistence with cell densities of up to 10^6–10^7 cells cm^{-2} (Vorholt, 2012). Within the diverse leaf microbial communities, Proteobacteria species constitute approximately half of the total phyllosphere community. However, our understanding of the specific roles and mechanisms of action of phyllosphere-associated PGPB remains an area with significant knowledge gaps, highlighting the need for further exploration and investigation in this important ecological niche.

1.4. *Paraburkholderia dioscoreae*

In 2020, Herpell *et al*., presented a novel *Paraburkholderia* isolate, named *Paraburkholderia dioscoreae*, strain Msb3. This isolate is a dominant and persistent member of the phyllosphere of *Dioscoreae bulbifera* and constitutes a substantial portion, up to 25%, of the microbial community residing on the leaf acumens.

The Msb3 genome is approximately 8.5 megabase pairs (Mbp) in size, consisting of two chromosomes and three plasmids, and exhibits an extraordinary combination of features that contribute to its beneficial association with plants.

When applied to agriculturally significant plants like tomatoes, the Msb3 isolate demonstrates a remarkable ability to promote plant growth by enhancing total dry biomass by up to 40%.

This finding highlights the substantial potential of the Msb3 isolate as a valuable candidate for promoting plant growth and establishing beneficial plant-microbe interactions, offering exciting prospects for agricultural applications (Herpell *et al*., 2020).

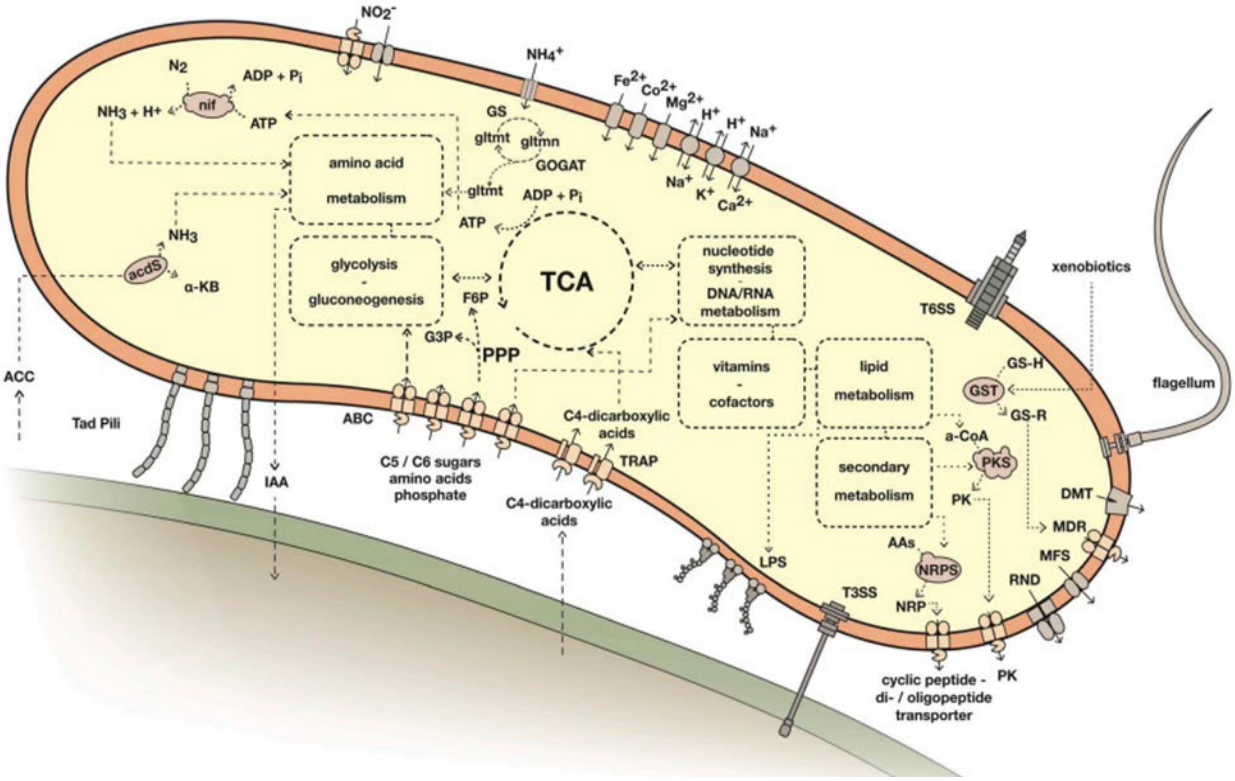

FIGURE 1 | ***Paraburkholderia dioscorea* strain Msb3**. A schematic representation of metabolic reconstruction, using the genomic sequence as a foundation. The image was adapted from Herpell *et al*. (2020) in *Front. Microbiol*. 11:581.

Among notable features of Msb3 that may contribute to its growth-promoting effect on host plants, is the presence of *acdS* gene. This gene encodes an enzyme called ACC deaminase, which plays a role in reducing the levels of ethylene in plants by cleaving the plant-produced ACC, a precursor of ethylene.

Ethylene is a plant hormone that can stimulate plant growth and development, as well as inhibit the plant from growing and lead to the premature senescence. In addition to its role in normal plant development, ethylene can also be involved in the plant's response to environmental stress, physical damage and other stress-related responses. Overall, the effect of ethylene on plants depends on the levels of the hormone present (Prat and Goeschl, 1969).

By decreasing ethylene levels, the plant becomes more resilient to environmental stresses. Therefore, the production of ACC deaminase by Msb3 has the potential to promote plant growth by modulating ethylene levels.

In addition, another mechanism through which plant-associated bacteria can enhance plant growth is by providing nutrients, particularly nitrogen. In the case of Msb3, it carries a complete nitrogenase gene cluster. This genetic feature suggests that Msb3 has the capacity

for biological nitrogen fixation, which could directly or indirectly contribute to plant growth by supplying nitrogen to the plant.

1.5. Aim of the study

Microbiota research faces a significant challenge due to the vast diversity of symbiotic bacteria. While DNA sequencing-based studies provide valuable correlations, there is a need to dive deeper into understanding the cellular and molecular mechanisms through which these bacteria impact their hosts. But progress in this area has been hindered by the slow and challenging nature of genetic manipulation, especially for new and uncharacterized bacterial isolates, using existing genetic tools.

Being a novel isolate, Msb3 lacks an established genetic system. Therefore, the primary objective of this research was to establish a genetic system for *Paraburkholderia dioscoreae*, strain Msb3 and lay the foundation for future studies on its molecular mechanisms.

However, developing new tools from scratch is a demanding and time-consuming task, in any novel bacterial species, especially in one with such a large genome like Msb3. For this reason, an allelic exchange system that was specifically developed to expand the possibilities of genetic manipulation in *Burkholderia* genus, was used (Hamad et al., 2008).

To investigate the extent to which the notable features of Msb3 contribute to the beneficial interactions with its host plant, the targeted genes, namely *nifH* and *acdS*, were knocked out. Subsequently, the tomato seedlings were inoculated with each respective KO mutant, and the resulting phenotypes were carefully observed and the colonization ability carefully analyzed. By comparing the characteristics of the plant inoculated with wildtype strain (with intact nifH and ACC-deaminase genes) to those inoculated with the KO mutants, insights were gained into the mechanisms through which Msb3 promotes plant growth.

The employed methods and the achieved results are presented in the following sections.

2. Materials

2.1. Bacterial strains, growth conditions, and culture media

All bacterial strains used in this study are listed in Table 1. Unless otherwise stated, *Paraburkholderia* were grown in liquid LB broth (pH 7) at 28°C and 170 RPM, or on LB agar plates at 28°C. *E. coli* was grown at 37°C 170 RPM in liquid LB broth or LB agar plates and kanamycin was used at 50 µg/ml or 100 µg/ml for plasmid selection. Exception was strain WM3064 that was grown at 31°C 170 RPM in liquid LB broth or LB agar plates containing 0.3 mM diaminopimelic acid (DAP), with 50 µg/ml for plasmid selection.

TABLE 1 | Strains used in the present study.

Strains	Relevant features	Reference
Paraburkholderia dioscoreae Msb3	Leaf colonizer, PGP	Herpell et al., 2021
Paraburkholderia phytofirmans PsJN	Plant colonizer, PGP	Donation of Birgit Mitter (AIT, Tulln, Austria)
E. coli WM3064	Biparental mating, donor of pMo130 derivates	Addgene
E. coli TOP10	Cloning, vector propagation	Thermo Fischer Scientific
E. coli S17-1	Biparental mating, donor of pMRE-Tn5-15x	Addgene
E. coli JM109	vector propagation	Addgene

2.2. DNA methods, PCR, and cloning

Chromosomal *Paraburkholderia* DNA was extracted following a standard phenol/chloroform/isoamyl alcohol (PCI) extraction protocol. DNA was amplified using Phire Hot Start II Polymerase (Thermo Fisher Scientific) in 20µl reaction mix consisted of 11.7µL sterile ddH_2O, 4 µL 5 x Phire Green Reaction Buffer (1.5 mM $MgCl_2$ final concentration), 0.4 µL dNTP Solution Mix (10 mM of each dNTP), 0.3 µL Phire Hot Start II Polymerase and 1 mL 10 mM forward and 1 mL 10 mM reverse primer as well as 1 µL template DNA (1ng/µL) or a colony lysed in sterile ddH2O. PCR products were purified using Monarch® PCR & DNA Cleanup Kit or Monarch® DNA Gel Extraction Kit (New England Biolabs). Primers were purchased from Microsynth (Microsynth AG, Balgach, Switzerland) A list of all the primer sequences used in this study is presented in Table 2. Plasmid DNA was isolated using PureYield™ Plasmid Miniprep System (Promega). List of all the plasmids used in this study is presented in Table 3. Restriction enzymes and T4 DNA ligase were purchased from NEB and used as outlined by the manufacturer (New England Biolabs).

TABLE 2 | Oligonucleotides used in the present study.

Target group	Name	Target accession	Target site	Sequence
Univ. Bacteria	27F	na	na	AGAGTTTGATCCTGGCTCAG
Univ. Bacteria	1492R	na	na	CTACGGCTACCTTGTTACGA
***Burkholderia spp.* sensu lato**	gyrB_F	na	na	ACCGGTCTGCAYCACCTCGT
***Burkholderia spp.* sensu lato**	gyrB_R	na	na	YTCGTTGWARCTGTCGTTCCACTGC
***Paraburkholderia dioscoreae* Msb3**	ΔacdS_US_f1	NZ_LR699554	1964236-1964255	CGTTACTACGCACGGCATGT
***Paraburkholderia dioscoreae* Msb3**	ΔacdS_US_r1	NZ_LR699554	1965186-1965167	AGGGAGAACCGCCAGAAATG
***Paraburkholderia dioscoreae* Msb3**	ΔacdS_DS_f1	NZ_LR699554	1966395-1966414	GCTTCCTCAAGTGCTCCCTC
***Paraburkholderia dioscoreae* Msb3**	ΔacdS_DS_r1	NZ_LR699554	1967359-1967340	GCGACCCATGAAACGAAGTG
***Paraburkholderia dioscoreae* Msb3**	ΔacdS_US_NheI	NZ_LR699554	1964236-1964255	gtcgtcagtagctagcctcgCGTTACTACGCACGGCATGT
***Paraburkholderia dioscoreae* Msb3**	ΔacdS_US_BglII	NZ_LR699554	1965186-1965167	gctagtcagcaagatctggtgAGGGAGAACCGCCAGAAATG
***Paraburkholderia dioscoreae* Msb3**	ΔacdS_DS_BglII	NZ_LR699554	1966395-1966414	gctgctgacgctagatctcagaGCTTCCTCAAGTGCTCCCTC
***Paraburkholderia dioscoreae* Msb3**	ΔacdS_DS_HindIII	NZ_LR699554	1967359-1967340	gtacgatcgacaagcttcggtGCGACCCATGAAACGAAGTG
***Paraburkholderia dioscoreae* Msb3**	acds_KO_control_F	NZ_LR699554	1964172-1964193	CGAAGAGAGGGTTATGTTTCGC
***Paraburkholderia dioscoreae* Msb3**	acds_KO_control_R	NZ_LR699554	1967397-1967376	TTCTCCTTGAGTTGTGCGATCC
***Paraburkholderia dioscoreae* Msb3**	acds_F1	NZ_LR699554	1965632-1965651	GTATCCGACGGCTTCGACAT
***Paraburkholderia dioscoreae* Msb3**	acds_R1	NZ_LR699554	1965824-1965824	CGTAGTCGAACCTGAAGCCC
***Paraburkholderia dioscoreae* Msb3**	nifH_US_F1	NZ_LR699554	1691826-1691845	CGCAGGCTCTTCTCGATCAT
***Paraburkholderia dioscoreae* Msb3**	nifH_US_R1	NZ_LR699554	1692801-1692782	CGCGAGGCCTGATATTCACC
***Paraburkholderia dioscoreae* Msb3**	nifH_DS_F1	NZ_LR699554	1693813-1693832	CTGTCGTGCCTGTATGGGTC
***Paraburkholderia dioscoreae* Msb3**	nifH_DS_R1	NZ_LR699554	1694840-1694822	CCTGATCGCAGGTCAGCTC
***Paraburkholderia dioscoreae* Msb3**	nifH_US_F1_NheI	NZ_LR699554	1691826-1691845	CGGCTAGCCGCGCAGGCTCTTCTCGATCAT
***Paraburkholderia dioscoreae* Msb3**	nifH_US_R1_BglII	NZ_LR699554	1692801-1692782	GAAGATCTTCCGCGAGGCCTGATATTCACC
***Paraburkholderia dioscoreae* Msb3**	nifH_DS_F1_BglII	NZ_LR699554	1693813-1693832	GAAGATCTTCCTGTCGTGCCTGTATGGGTC
***Paraburkholderia dioscoreae* Msb3**	nifH_DS_R1_HindIII	NZ_LR699554	1694840-1694822	CCCAAGCTTGGGCCTGATCGCAGGTCAGCTC
***Paraburkholderia dioscoreae* Msb3**	nifH_F	NZ_LR699554	1693194-1693175	ACGAACGTCAGACCGACAAG
***Paraburkholderia dioscoreae* Msb3**	nifH_R	NZ_LR699554	1693014-1692995	CGCGTTGTTGTGTACCTTGG

TABLE 3 | Plasmids used in the present study.

Plasmids	Relevant features and use	Reference
pMo130	KmR, sacB, MCSI, oriT; allelic exchange suicide vector backbone	Hamad et al., 2009
pAJ101	pMo130 derivative for acdS KO	present study
pAJ102	pMo130 derivative for nifH KO	present study
pMRE-Tn5-152	sGFP2, KmR, NeoR, CmR, AmpR, oriT; Tn5 transposase based chromosomal integration	Schlechter et al., 2018
pMRE-Tn5-155	mScarlet-I, KmR, NeoR, CmR, AmpR, oriT; Tn5 transposase based chromosomal integration	Schlechter et al., 2018

2.3. Tomato seeds and growth medium

Moneymaker Tomato seeds (*Lycopersicum esculentum*) were usedfor all growth promotion assays and Murashige & Skoog (MS) medium (Duchefa Biochemie B.V, Haarlem, Netherlands) was used for tomato seed germination and growth promotion assays.

3. Methods

3.1. *Paraburkholderia dioscoreae* Msb3 knockout constructions

Paraburkholderia dioscoreae Msb3 knockouts were constructed using allelic exchange. This method relies on homologous replication and allows for the replacement of a specific DNA segment of bacterial genome with a modified DNA sequence of choice. The replacement done in the case of knockouts constructed in this study was in a form of deletion.

Allelic exchange requires homologous DNA to start the process of recombination; therefore, the first step in constructing a deletion mutant with allelic exchange is to amplify upstream and downstream fragments of the gene of interest. Since the efficiency of recombination is influenced by the length of the DNA fragments employed, fragments of approximately 1000bp are recommended.

3.1.1. Homologous flanks amplification

DNA extracted from *Paraburkholderia dioscoreae* strain Msb3 was used as a template for homologous flanks amplification.

US and DS fragments flanking the acds gene were amplified using primers ΔacdS_US_f1 and ΔacdS_US_r1, ΔacdS_DS_f1 and ΔacdS_DS_r1 respectively. Both US and DS fragments were amplified in following conditions: one cycle of initial denaturation at 98˚C (3 min); 30 cycles: denaturation 98˚C (10 s), primer annealing 60˚C (15 s), extension 72˚C (10 s); one cycle final extension 72˚C (10 s). Resulting PCR products (~1kb long) were purified and used as a template for the next PCR. This amplification was done with primers that amplify US fragment flanked by NheI and BglII recognition sites, as well as DS fragment flanked by BglII and HindIII recognition sites. Primers used were ΔacdS_US_NheI and nifH_US_R1_BglII for US, nifH_DS_F1_BglII and nifH_DS_R1_HindIII for DS fragment. Reaction conditions for both US and DS fragments were as follows: one cycle initial denaturation at 98˚C (3 min); 30 cycles: denaturation 98˚C (10 s), primer annealing at 72˚C (15 s), extension 72˚C (10 s). Resulting PCR products were purified. The US fragment was digested with NheI-HF and BglII, while the DS fragment was digested with BglII and HindIII-HF and both were ligated with Quick Ligase. Ligation reaction was then purified and PCR amplified in following conditions: one cycle at 98˚C (3 min); 30 cycles at 98˚C (10 s), 72˚C (15 s), 72˚C (21 s); one cycle final extension at 72˚C (60 s). Amplification resulted in a product that was ~2kb long.

US and DS fragments flanking the nifH gene were amplified and ligated in the same manner. First round of PCR with primers nifH_US_F1 and nifH_US_R1 for US fragment, as well as

nifH_DS_F1 and nifH_DS_R1 for the DS fragment was done in following conditions: one cycle at 98˚C (3 min); 30 cycles at 98˚C (10 s), 60˚C (15 s), 72˚C (10 s); one cycle final extension 72˚C (10 s). In the second round of PCR, following primers with restriction motifs were used: nifH_US_F1_NheI and nifH_US_R1_BglII, nifH_DS_F1_BglII and nifH_DS_R1_HindIII. Reaction conditions for both US and DS fragment: one cycle at 98˚C (2 min); 30 cycles at 98˚C (10 s), 72˚C (15 s), 72˚C (10 s); one cycle final extension 72˚C (20 s). Ligated products were amplified with nifH_US_F1_NheI and nifH_DS_R1_HindIII in following conditions: one cycle at 98˚C (3 min); 30 cycles at 98˚C (10 s), 72˚C (15 s), 72˚C (20 s); one cycle final extension 72˚C (20 s).

3.1.2. Knockout suicide vector pAJ10x construction

For construction of pAJ10x plasmid, pMo130 was used as an empty vector backbone. pMo130 was purchased from Addgene together with *E. coli* JM109. This strain was grown in 30°C in 50 µg/ml kanamycin and plasmid was isolated as mentioned in section 1.2.

pMo130 was linearized with NheI-HF and HindIII-HF restriction endonucleases. Ligated 2kb homologous flanks of acds and nifH (mentioned in the 2.1.1. section) were also digested with NheI-HF and HindIII-HF and then ligated together with pMo130, to generate pAJ101 or pAJ102.

Vectors were mobilized into *E.coli* WM3064 via heat shock transformation and maintained at 31 °C on LB containing 50 µg/ml kanamycin and 0.3 mM diaminopimelic acid (DAP).

3.1.3. Conjugative transfer of pAJ10x into *Paraburkholderia dioscoreae* Msb3

Biparental mating was performed with *E. coli* WM3064 and Msb3. Msb3 and *E. coli* WM3064 containing pAJ10x were grown as explained in section 1.1. Msb3 was harvested at an OD_{600} > 0.7 and E. coli WM3064 at an OD_{600} of 0.5. Both strains were separately washed 2 times with 1 × LB medium and then mixed in 1:1 ratio. The mixture was then centrifuged and resuspended in ~1/10 the volume and drop spotted in a single pool on LB agar containing 0.3 mM DAP, followed with an incubation step at 28 °C overnight. After the incubation, exconjugants were streaked onto 1 × LB or M9 minimal medium with glucose plates lacking DAP and containing 100 µg/ml kanamycin to select for Msb3 that had completed the first crossover. First crossover strains were then re-streaked three times to be purified from donor strain.

3.1.4. Resolution of pAJ10x integration and knockout strain purification

To resolve the pAJ10x incorporation, first crossover strains were cultivated in LB medium containing 100 g/ml kanamycin. They were then plated on YT medium, containing 10% sucrose (10 g/l tryptone, 5 g/l yeast extract, 100 g/l sucrose, and 1% agar). Once colonies were big enough, they were picked and cultivated in the same liquid medium. To screen for deletion, various PCRs were performed on resulting strains.

To confirm that acds gene was deleted two PCRs were performed, using acds_KO_control_F and acds_KO_control_R, as well as acdSF1 and acdSF1R1 primers. The first pair of PCR primers was created to sequence across the deleted region and was done in following conditions: one cycle at 98˚C (3 min); 30 cycles at 98˚C (10 s), 60˚C (15 s), 72˚C (2 s). The second pair of PCR primers was designed to amplify a 200 bp long region of the gene itself and was done in following conditions: one cycle at 98˚C (3 min); 30 cycles at 98˚C (10 s), 60˚C (15 s), 72˚C (32 s).

For nifH deletion confirmation, a PCR that will amplify a 200 bp long region of the nifH gene was performed, using the nifH_F and nifH_R primers. Conditions were as follows: one cycle at 98˚C (3 min); 30 cycles at 98˚C (10 s), 60˚C (15 s), 72˚C (2 s). To sequence throughout the deleted area, primers nifH_US_F1 and nifH_DS_R1 were used in the PCR with following conditions: one cycle at 98˚C (3 min); 30 cycles at 98˚C (10 s), 60˚C (15 s), 72˚C (31 s).

To ensure the purity of each KO strain a PCR was performed, using primers that will amplify a *Burkholderia spp*. gyrase B. Primers gyrB_F and gyrB_R were used and PCR conditions were as follows: one cycle at 98˚C (2 min); 30 cycles at 98˚C (10 s), 60˚C (10 s), 72˚C (8 s).

For allof the above mentioned PCRs, Msb3 wt DNA was used as control.

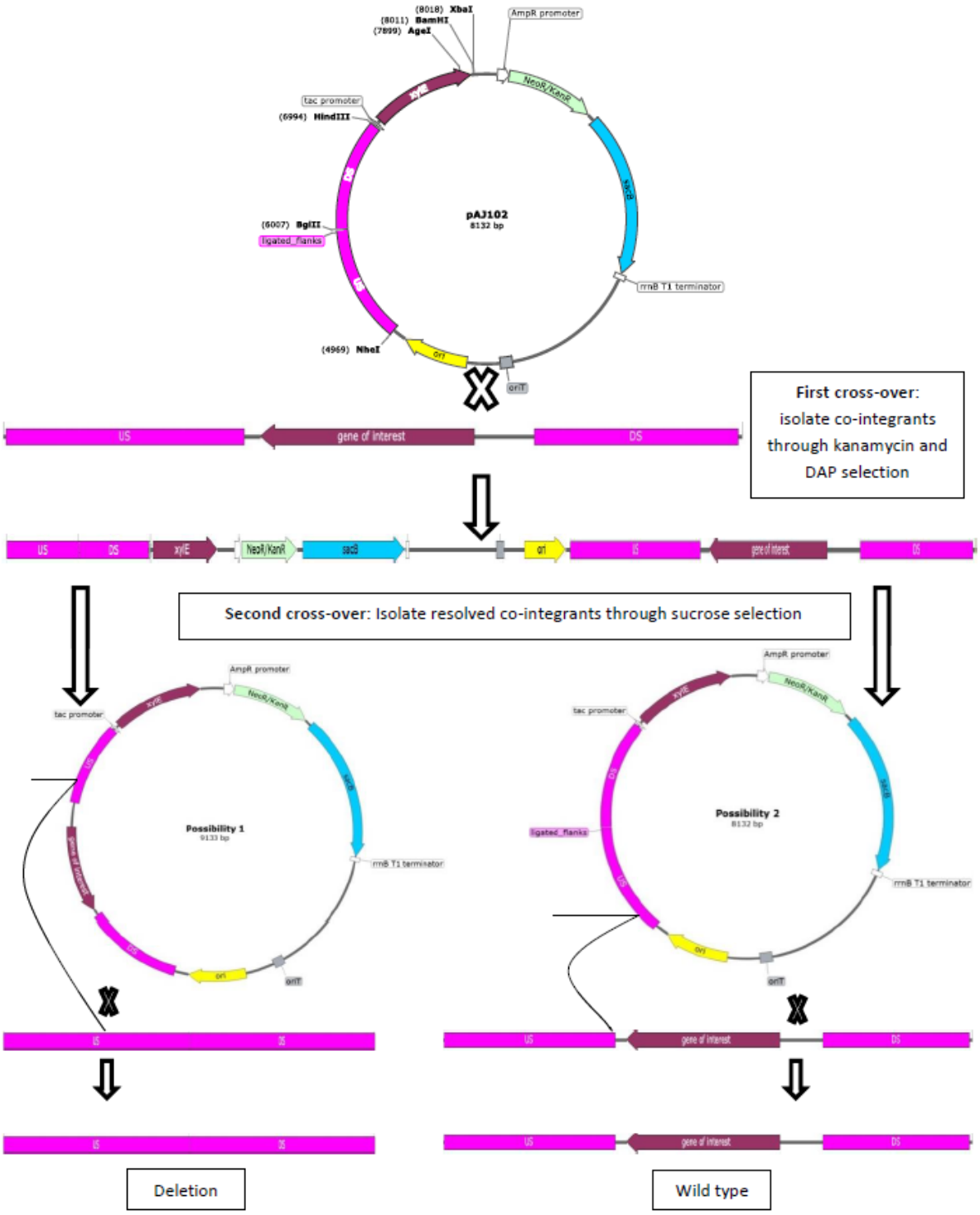

FIGURE 2 | **A schematic representation of the allelic exchange system, used for generation of KO mutants in *P.dioscoreae.*** To initiate the process, DNA fragments located upstream and downstream of the target gene are inserted into pMo130, resulting in the creation of pAJ101/pAJ102. Subsequently, the constructed pAJ0x plasmid is transferred into *P. dioscoreae* via conjugation, and co-integrants are isolated through kanamycin and DAP selection. The next step involves the retrieval of resolved co-integrants using sucrose counter-selection. To differentiate between deletion mutants and wild-type isolates, colonies are subjected to PCR analysis.

3.2. Construction of fluorescently tagged *Paraburkholderia* strains

We created fluorescently tagged bacterial strains that were resistant to kanamycin via stable chromosomal insertions via a Tn5 transposon-based delivery system [59]. Both plasmids were delivered in *E. coli* strain S17-1 that allows conjugation of the mobilisable plasmids into other bacteria. *E. coli* strain S17-1 was grown in 1 × LB containing 50 µg/ml ampicillin at 30°C to an OD_{600} of 0.3. Recipients were grown in 1 × LB without antibiotics to an OD_{600} of around 0.7. Each strain was washed separately two times with 1 × LB medium, then they were mixed at ratios 1:1, centrifuged and resuspended in approximately 1/10 the volume and plated in a single pool on 1 × LB agar. The mixture was incubated at 28°C overnight. Exconjugants were streaked onto M9 minimal medium agar plates containing glucose or malic acid as sole carbon source and 100 µg/ml kanamycin. After 3 to 5 days the colonies that appeared were re-streaked on the same media. 3 rounds of re-streaks were performed to make sure the strains were pure.

3.2.1. Screening of fluorescently tagged strains

Fluorescently tagged strains were screened for their ability to grow normally in rich medium. If a strain displayed impaired growth it was no used in subsequent re-cultivation experiments. Fluorophore expression was checked on a GelDoc Go Imaging System on a UV/Stain-Free Tray (Bio-Rad) . If there was no growth deficiency and proper fluorophore expression one eGFP2 and one mScarlet expressing clone of each strain was chosen for downstream experiments. The GelDoc Go Imaging System was equipped with filters for fluorescence of various commercial dyes. A culture of Msb3 wt was used as negative control and the *E. coli* donor strains were used as positive controls to create reproducible settings. Of each plate one brightfield image was obtained (exposure for 0.5 s) as well as one image through a filter for Atto488 (0.3 s) and one through a filter for Cy3 (4 s).

TABLE 4 | Fluorescently tagged plasmids used in the present study.

Name	Acronym	Plasmid	Excitation max	Emission max	Scann
sGFP2		**pMRE-Tn5-152**	495 nm	512 nm	**2**
mScarlet-I	(mSc)	**pMRE-Tn5-155**	560 - 575 nm	585 - 610 nm	**1**

3.2.2. Re-cultivation assay setup

Re-cultivation experiments were conducted using the *in vitro* growth system for tomato. Fluorescently tagged strains were grown to an OD_{600} of 0.6 in TSB containing 100 µg/ml kanamycin. Cells were washed twice in 10 mM $MgCl_2$. Plants were inoculated as described before. Plants were grown for seven days after inoculation. Upon harvest, the plant was removed from the container, shoot and root systems were separated and residual agar was removed from the roots by washing with glass beads in 0.4% NaCl. Using sterile mortar and pestle the tissue was carefully ground in 1 ml 0.4% NaCl. The liquid was recovered and used to create serial dilutions. 5 µl were plated onto M9 minimal medium agar containing 20 g/l glucose and 100 µg/ml kanamycin in a dropwise fashion. After 3 days the plates were imaged outlined above and the resulting images were used to quantify CFUs.

3.3. Msb3ΔacdS and Msb3ΔnifH growth promotion assays in tomato

3.3.1. Setup

Tomato seeds were surface sterilized by washing with 70% ethanol 3 times for 10 minutes, washed in sterile distilled water 3 times for 10 minutes and placed onto 12 × 12 cm plates with 15mL Murashige & Skoog (MS) agar medium. Seeds were then germinated at 25˚C for 5 days in dark, followed by 2 days of light exposure.

Uniform 7-day old Tomato seedlings were inoculated with bacterial suspensions and grown in jam jars that were randomly placed in a growth chamber with a photoperiod of 16 h at 25 °C during the day and 18 °C during the night and a relative humidity of 60%.

3.3.2. Bacterial culture and plant inoculation

Msb3 wildtype, the *acdS* deficient strain Msb3ΔacdS and the *nifH* deficient strain Msb3ΔnifH were grown in tryptic soy broth at 28˚C and 170RPM. They were harvested at an OD_{600} of 0.6 and washed 3 times in 10 mM $MgCl_2$. A bacterial solution with an OD_{600} of 0.01 in 10 mM $MgCl_2$ was used for inoculation. Inoculated seedlings, as well as non-inoculated controls, were grown on 15mL MS agar in jam jars and for certain experiments, 400 nM ACC was added to the plant culture media.

3.3.3. Growth promotion assay

Inoculated tomato seedlings were grown for 7 days in a growth chamber under the same conditions as the seeds (section 2.2.1.). After 7 days all tomato plantlets were removed from jam jars for phenotypic evaluation. The lengths of hypocotyl and primary root were determined via image analysis, while the number and cumulative length of the side roots were determined manually.

3.3.4. Root and shoot image analysis

Plants were imaged using a fixed Nikon camera and a scale was included in each image. Primary root length elongation and hypocotyl length was measured using ImageJ.

3.3.5. Phenotypic analyses

Phenotypic parameters were compared across the no bacteria, Msb3 and Msb3ΔacdS treatments using a two-sided ANOVA model. Normal distribution was assumed. Differences between treatments were indicated using the confidence letter display derived from the Tukey's post hoc test implemented in the package agricolae.

3.3.6. $15N_2$ Labeling experiments in the tomato phyllosphere: Msb3 wt vs. Msb3ΔnifH

Tomato seedlings were cultivated in vitro using Schott-bottles sealed with rubber lids. Prior to labeling, the plants were subjected to different inoculations: no bacteria control, Msb3 wild-type or Msb3ΔnifH. The atmosphere within the bottles was substituted with an artificial atmosphere consisting of around 20% $15N_2$ labeled gas, while maintaining the pressure inside at atmospheric levels. After a two-day period, another atmosphere exchange took place. Subsequently, the plants were harvested after four days, dried, and their d15N/14N ratio was determined through isotope ratio mass spectrometry (IR-MS).

3.4. In vitro cultivation of Msb3 and Msb3ΔacdS on ACC

Both strains were initially cultured from cryostock Tryptic Soy Broth. Overnight growth was followed by thoroughly washing both cultures 3 times with 10 mM $MgCl_2$ solution. Then, M9 minimal media with various sources of carbon or nitrogen was inoculated with a minimal number of the washed cells (OD600 < 0.001), to ensure accurate results and prevent cell debris from serving as a nutrient source. M9 minimal medium, supplemented with 2 µM glucose as the carbon source and 1 µM ammonia as the nitrogen source, was used as a positive control. Negative controls were established under the following conditions: M9 salts only, M9 salts with 2 µM glucose, and M9 salts with 2 µM ammonia. To examine growth on ACC (aminocyclopropane-1-carboxylate), three different conditions were tested:

1. ACC as the carbon source (M9 salts, 2 µM ACC, 1 µM ammonia);
2. ACC as the nitrogen source (M9 salts, 1 µM ACC, 2 µM glucose);
3. ACC as both the carbon and nitrogen source (M9 salts, 2 µM ACC).

4. Results

Genomic analyses of Msb3 have revealed potential mechanisms that may contribute to its growth-promoting effect on host plants. One notable feature is the presence of genes involved in plant growth promotion, including the *nifH* and *acdS* gene. This study was focused on developing a genetic system in beta with the aim to unravel these specific mechanisms by which Msb3 facilitates beneficial interactions and promotes plant growth and shed light on the significance of *nifH* and ACC-deaminase genes in this process. Results are presented in the following sections.

4.1. Msb3ΔnifH

4.1.1. *nifH* knockout construction: confirmed deletion of *nifH* gene in Msb3

The use of allelic exchange system in *P. dioscoreae* demonstrated a high level of effectiveness, resulting in the successful generation of a *nifH* KO mutant with the targeted deletion of the *nifH* gene. PCR analysis was performed to evaluate the presence of the desired genetic modification. Amplification of the 3 kb fragment, consisting of the *nifH* gene and the flanking fragments was performed using the nifH_US_F1 and nifH_DS_R1 primers. It revealed a distinctive band 1012 bp smaller than the Msb3 wild-type. The conjugational

donor (E. coli WM3064) of the knockout vector was used as a negative control, while the recipient Msb3 (wt) DNA was used as a positive control, confirming the expected band size. Furthermore, amplification of the *nifH* gene using Ueda19F and R6 primers showed that the *nifH* knockout DNA failed to yield any amplification, while the Msb3 wild-type DNA that was used as a positive control resulted in the desired size of the amplicon. These findings confirmed the successful deletion of the *nifH* gene in the constructed *nifH* mutant (Figure 3). Msb3ΔnifH was also successfully fluorescently tagged with both GFP2 or mScarlet-1.

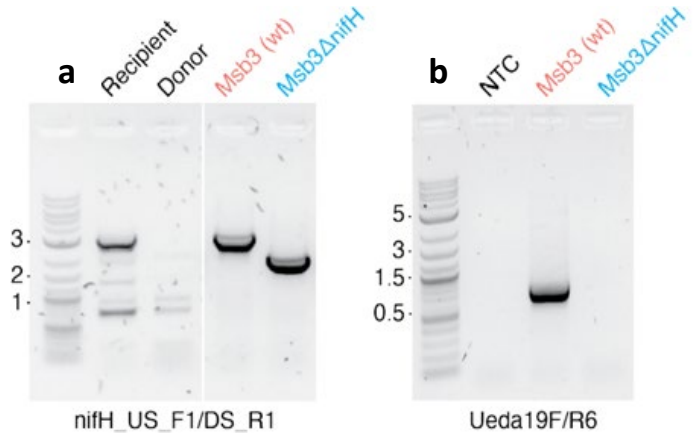

FIGURE 3 | **PCR results confirming the deletion of the *nifH* gene. a)** The 3 kb fragment encompassing the *nifH* gene and flanking regions was amplified using nifH_US_F1 and nifH_DS_R1 primers. The conjugational donor (E. coli WM3064) was used as a negative control, and the recipient Msb3 (wt) DNA wasused as a positive control. The 3kb band was amplified from the wild-type Msb3 DNA, while the band amplified from the nifH knockout DNA was 1012bp smaller. **b)** Amplification of the *nifH* gene using Ueda19F and R6 primers exhibited a band of 1012 bp, the desired size of the Msb3 wild-type DNA . The *nifH* knockout demonstrated the absence of amplification, thus confirming the successful deletion of the *nifH* gene in the constructed *nifH* mutant. Figure adapted from Herpell *et al*. (2023).

4.1.2. Knocking out *nifH* has no effect on plant colonization and growth promotion properties of Msb3

The deletion of *nifH* gene, did not exhibit any impact on either plant colonization or growth promotion properties of Msb3. This observation aligns with results of $15N_2$ labeling experiments, which indicated the absence of nitrogen fixation in our in vitro conditions (Figure 4). Based on these findings, it can be concluded that the observed plant growth promotion effect is not attributed to nitrogen supply, at least under these circumstances.

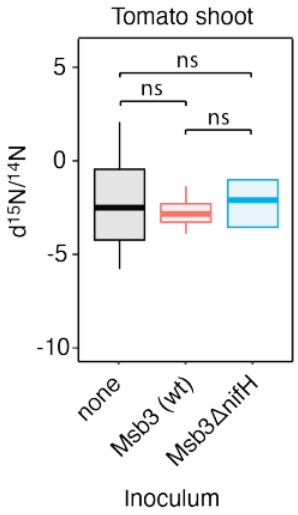

FIGURE 4 | Results of $15N_2$ Labelling Experiments in the Tomato Phyllosphere: Msb3 wt vs. Msb3ΔnifH: Performed as explained in Methods section 3.2.6. The comparison of treatment groups (n=4;4;3) did not reveal any significant differences. Significance between treatments was assessed using an ANOVA followed by a Tukey post hoc test. Figure adapted from Herpell *et al*. (2023).

4.2. Msb3ΔacdS

4.2.1. *acds* knockout construction: deletion of the *acds* gene in Msb3

The *acdS* knockout mutant was generated using the allelic exchange system described previously. The *acdS* gene deletion in the resolved exconjugants was assessed through PCR analysis.

First of all, a PCR using the gyrB_F and gyrB_R primers was performed. This pair of primers targets *Burkholderia spp*. *sensu lato* species. The *E. coli* donor strain WM3064, lacking pAJ101, was used as a negative control and did not produce any detectable bands when subjected to amplification with these primers. In contrast: the recipient strains Msb3 prior to conjugation, as well as the Msb3 wild-type DNA, were used as positive control and both produced a distinct and clear band. Following conjugation and allelic exchange, the exconjugant Msb3ΔacdS still produced the band of the same size. Further analysis through sequencing revealed that the amplified gyrase B fragment from Msb3ΔacdS was 100% identical to that of the Msb3 wild-type strain (Figure 5a).

A PCR analysis was conducted on the identical set of organisms, using primers acdS_KO_control_F/R. Primer acdS_KO_control_F binds ~1kb upstream of *acdS* and acdS_KO_control_R primer binds ~1kb downstream of the *acdS* gene. After the afore mentioned PCR, the presence of the 3 kb fragment, encompassing the *acdS* gene and

flanking regions, was observed in the wild-type. A distinct band, 1228bp smaller than the wild-type, was amplified from the *acdS* knockout mutant DNA, which corresponds to the size of between the two homologous flanks (Figure 5b).

To further prove deletion of the *acdS* gene, another PCR was performed using acdS_F1 and acds_R1 primers which amplify 193 bp of the Msb3 *acdS* gene itself. Upon amplification with these primers, the Msb3 wild-type DNA yielded a band of the expected size. In contrast, the *acdS* knockout mutant failed to produce any amplification. This absence of amplification in the *acdS* knockout DNA, while the positive control exhibited the expected band size, provided additional evidence of the successful deletion of the *acdS* gene (Figure 5c).

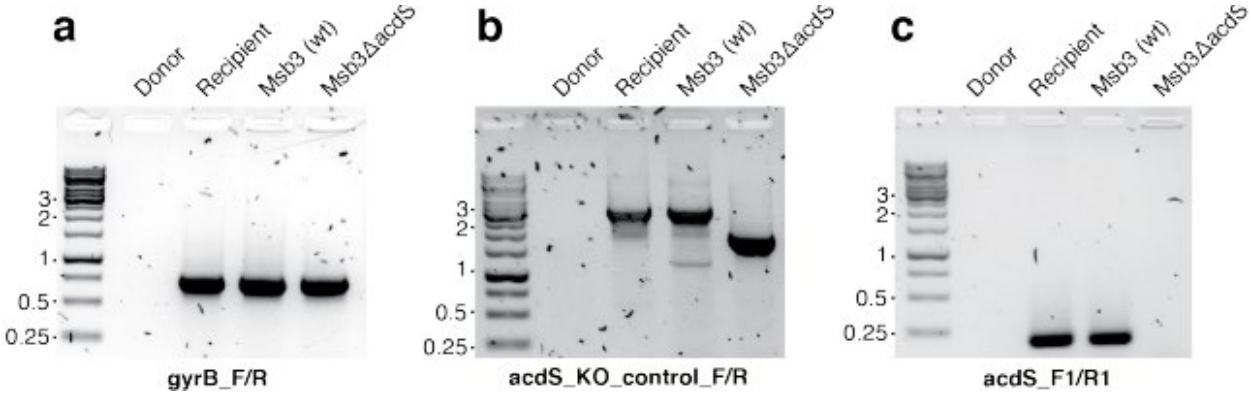

FIGURE 5 | **PCR results confirming the deletion of the *acdS* gene. a)** Amplification using gyrB_F and gyrB_R primers, which specifically target *Burkholderia spp. sensu lato* species. The conjugational donor (E. coli WM3064) was used as a negative control, while the pre-conjugation recipient Msb3, as wellas the Msb3 wild-type DNA were used as a positive control. Msb3ΔacdS displayed a clear band sized identically as the positive controls. Sequencing analysis confirmed that it was also 100% identical to that of the Msb3 wild-type strain. **b)** The 3 kb fragment encompassing the *acdS* gene and flanking regions was amplified from the wild-type Msb3 DNA, using acdS_US_F1 and acdS_DS_R1 primers. The band amplified from the *acdS* knockout DNA was 1228bp smaller, which is the size of the Msb3 *acdS* gene. **c)** Pimers acdS_F1 and acds_R1 amplify 193 bp fragment of the Msb3 *acdS* gene itself. The Msb3 wild-type DNA exhibited a band of 193 bp. The acdS knockout DNA demonstrated the absence of amplification, thus confirming the successful deletion of the *acdS* gene in the constructed mutant. Figure adapted from Herpell *et al*. (2023).

4.2.2. Fluorescently tagged *acdS* knockouts

Fluorescent tagging of *acdS* mutants with GFP2 or mScarlet-1 was successful. It allowed for their visualization both as liquid cultures under a confocal laser scanning microscope, and as colonies on the GelDoc Go Imaging System, as shown in the Figure 6 and 7.

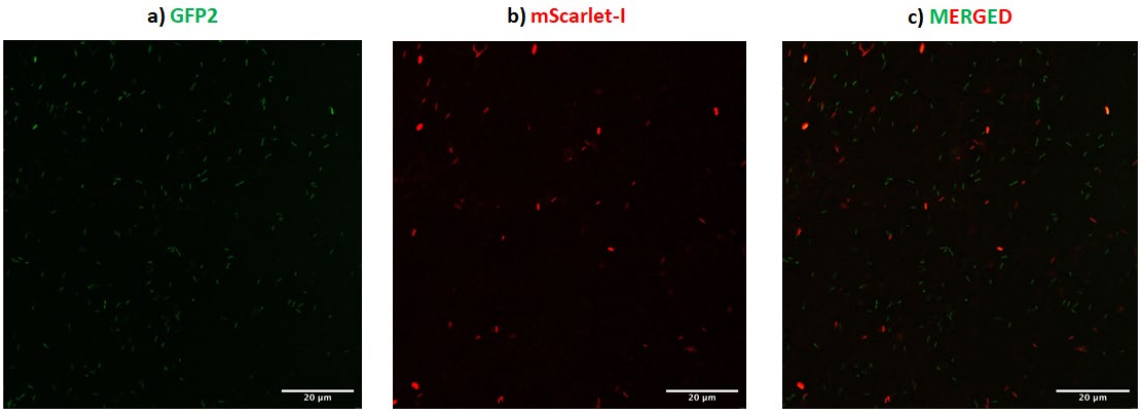

FIGURE 6 | **Liquid cultures of fluorescently tagged Msb3ΔacdS:** Confocal laser scanning microscopy images of liquid cultures of fluorescently tagged Msb3ΔacdS liquid cultures expressing **a)** GFP or **b)** mScarlet-I. **c)** A mixed culture of GFP2 and mScarlet-I expressing Msb3ΔacdS cells.

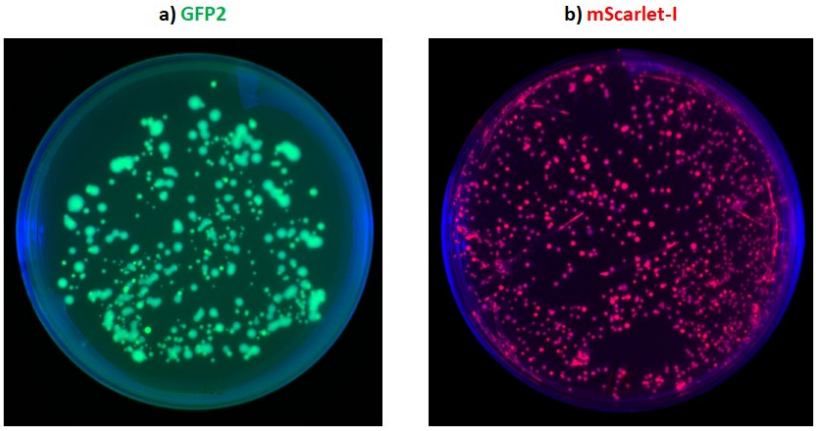

FIGURE 7 | **Colonies of fluorescently tagged Msb3ΔacdS:** ChemiDoc images of colonies of fluorescently tagged Msb3ΔacdS expressing **a)** GFP or **b**) mScarlet-I.

4.2.3. Deficiency of ACC deaminase in Msb3ΔacdS

As mentioned before, the *acdS* gene encodes for the ACC deaminase. ACC stands for aminocyclopropane-1-carboxylate and it is a precursor of the plant growth hormone ethylene. ACC deaminase cleaves the plant produced ACC and thereby can lower the levels of ethylene in the plant (Glick *et al*., 2007).

To test the ACC cleaving ability of both Msb3 wild-type and Msb3ΔacdS, they were grown in M9 minimal medium containing different nutrient sources. Since the wild-type Msb3 possesses an intact *acdS* gene, it implies that wild-type Msb3 also has the ability to utilize

ACC as the sole carbon or nitrogen source for growth. In contrast, the *acdS* KO mutant lacks the ability to cleave ACC, leading to its inability to utilize ACC as a carbon or nitrogen source. Consequently, the *acdS* KO mutant exhibited compromised growth in a medium where ACC is the only available source of those to elements (Figure 8). This observation provides clear evidence supporting the direct correlation between the deletion of the *acdS* gene and the incapacity of the Msb3ΔacdS to metabolize ACC.

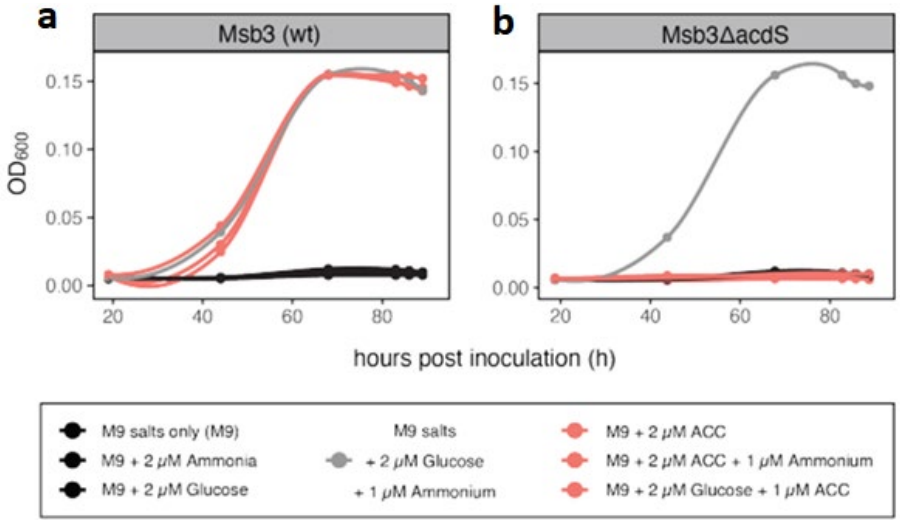

FIGURE 8 | **Deficiency of ACC deaminase in Msb3ΔacdS: a)** The growth of Msb3 wild-type and **b)** Msb3ΔacdS in M9 minimal medium supplemented with various nutrient sources. Negative controls, represented by black lines, lacked an essential nutrient source (n=1). Positive control, represented by grey lines, contained both carbon and nitrogen sources (n=1). The red lines indicate conditions where the medium was supplemented with ACC instead of either carbon (n=3), nitrogen (n=3), or both (n=3). Figure adapted from Herpell et al. (2023).

4.2.4. Knocking out *acdS* has no effect on plant colonization

To investigate the impact of *acdS* knockout on colonization and plant growth-promoting effects, re-cultivation assays were conducted in order to quantify colony-forming units as a measure of active cells. Fluorescently tagged Msb3 and Msb3ΔacdS strains, containing kanamycin resistance cassette for controlled conditions, were used in the assays (Figure 9). Re-cultivation was performed on M9 minimal medium supplemented with glucose as the sole carbon source, along with kanamycin for selective pressure. Results showed that the ability of Msb3ΔacdS to colonize and survive on the tomato shoot was not compromised. Both Msb3 wild-type and Msb3ΔacdS exhibited high CFU counts, reaching as high as 107 CFUs per tomato shoot (Figure 10). Additionally, it was observed that the abundance of Msb3 in leaves is approximately ten times higher compared to its abundance in roots, indicating a notable difference in colonization levels between these two plant tissues.

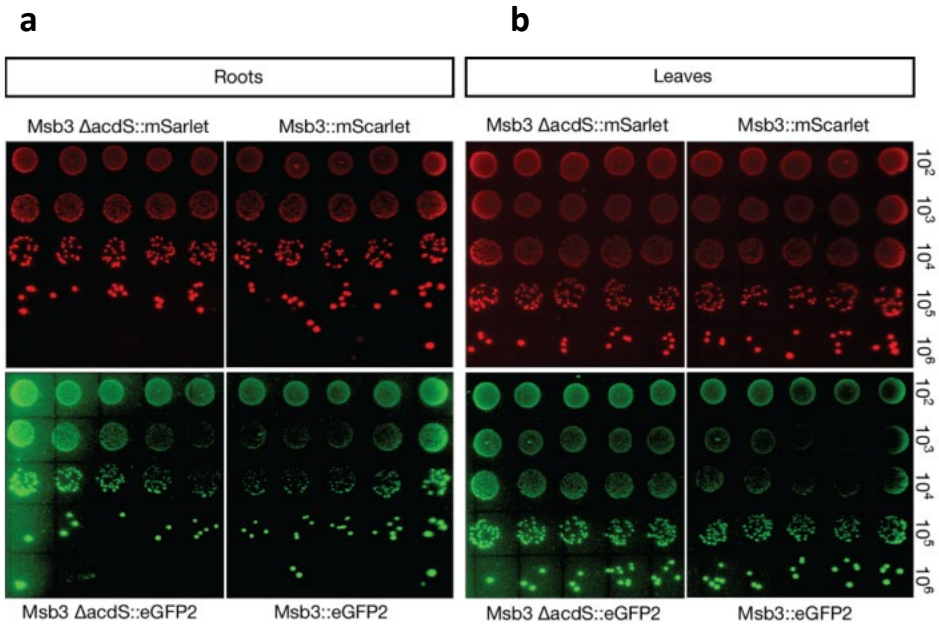

FIGURE 9 | **Recultivation assay of fluorescently tagged Msb3 wild-type and Msb3ΔacdS from tomato roots and leaves:** Re-cultivation assay performed to assess the presence of viable cells by plating serially diluted plant extracts of either **a)** roots or **b)** leaves on M9 minimal medium agar supplemented with glucose as the carbon source and kanamycin. Each row in the assay represents a tenfold serial dilution, indicated at the right side of the row. The columns correspond to different biological replicates inoculated with one of four mutant strains: Msb3ΔacdS derivative strains (Msb3ΔacdS::eGFP2 or Msb3ΔacdS::mScarlet) or Msb3 wildtype derivatives (Msb3::eGFP2 or Msb3::mScarlet). Figure adapted from Herpell et al. (2023).

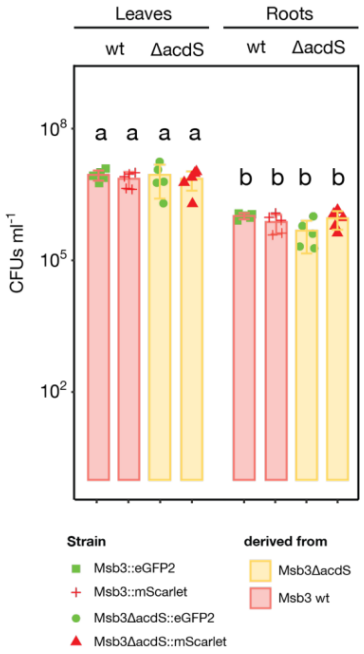

FIGURE 10 | **Quantification of Colony-Forming Units (CFUs) per ml in the Original Extract - Results of Figure 9:** In this analysis, serial dilutions of 105 (10^5) were employed to calculate the final values. Based on the assay, knocking out the *acdS* gene does not impact the ability of the Msb3 to colonize both leaf and root tissue. It was observed that Msb3 is approximately one order of magnitude more abundant in leaves compared to roots. Figure adapted from Herpell et al. (2023).

4.2.5. Msb3ΔacdS induced phenotypic alterations in tomato

During this study, it was observed that the lateral roots of plants inoculated with Msb3ΔacdS looked persistently different to those inoculated with the wild-type Msb3. So we decided to investigate the effects of different treatments on the formation and growth of lateral roots in tomato plants. The response of plants inoculated with Msb3ΔacdS to those inoculated with the wild-type Msb3 was compared. The results showed that plants inoculated with Msb3ΔacdS exhibited a significant difference in the number of lateral roots compared to plants inoculated with the Msb3 wild-type strain (Figure 11a). This indicates that the deletion of the *acdS* gene led to a change in lateral root development.

To further understand the mechanism behind lateral root formation, we analyzed the impact of adding ACC to the growth medium, since ACC is a precursor of ethylene, which is known to influence various physiological processes, including root development. Surprisingly, the addition of ACC did not affect the number of lateral roots (Figure 11b). This finding suggests that the stimulation of lateral root formation is not dependent on the hydrolysis of ACC in the rhizosphere, where the roots interact with the soil.

However, the treatment with ACC did have an effect on the length of the lateral roots (Figure 11c). This indicates that ACC in the growth medium inhibited the extension or elongation of the lateral roots while still allowing for their formation.

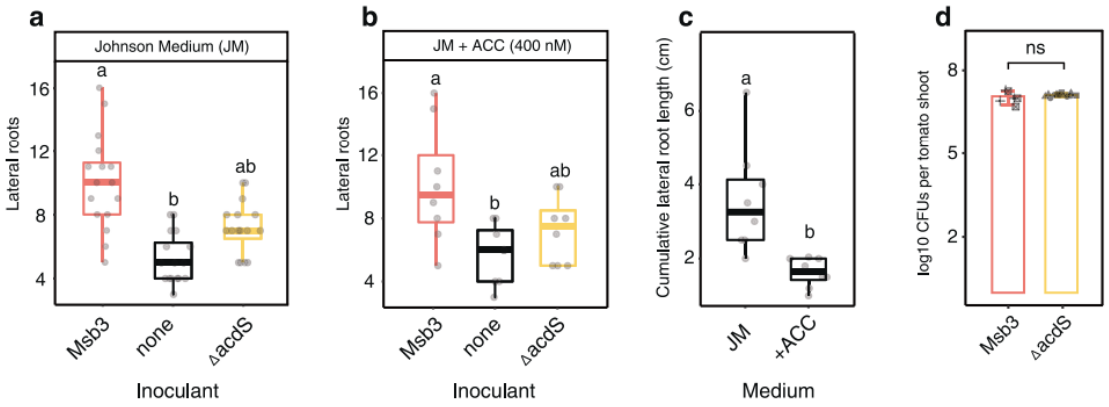

FIGURE 11 | **Phenotypic changes in Tomato roots induced by Msb3 or Msb3ΔacdS: a)** The number of first-degree side roots in tomato seedlings grown in Johnson medium (JM) at 7 days post-inoculation with Msb3, Msb3ΔacdS, or a non-inoculated control (n=16). **b)** The number of first-degree lateral roots in tomato seedlings grown in JM+ 400 nM of ACC at 7 days post-inoculation with Msb3, Msb3ΔacdS, or a non-inoculated control (n=8). **c)** Cumulative length of the first-degree side roots in non-inoculated samples grown in JM or JM+400 nM of ACC. Statistical significance in a),b) and c) was determined within each treatment using ANOVA, and the letters indicate the results of the Tukey post hoc test. **d)** Colony-forming units recovered from tomato shoots inoculated with fluorescently labelled Msb3 or Msb3ΔacdS. The red bar represents two Msb3 strains (n=3 each), while the yellow bar represents three Msb3ΔacdS strains (n=5 each). The shapes on the graph represent the respective derivative mutants: pluses indicate Msb3::eGFP2, checked boxes indicate Msb3::mScarlet,

circles indicate Msb3ΔacdS::eGFP2.1, triangles indicate Msb3ΔacdS::eGFP2.2, and squares indicate Msb3ΔacdS::mScarlet. Figure adapted from Herpell et al. (2023).

In summary, this experiment provided insights into the role of ACC in the regulation of lateral root development. The results suggest that ACC affects the length but not the formation of lateral roots, and that Msb3 is involved in promoting lateral root formation independently of ethylene signaling in the roots.

In order to obtain more detailed information about the characteristics of the plants, the lengths of the plantlets were measured, to assess the overall size and growth of the seedlings. Upon analyzing the data, a significant increase in the total seedling length of the plants inoculated with Msb3 compared to the non-inoculated control group was observed (Figure 12). This finding suggests that the presence of Msb3 in the plants positively influences the growth and development of the seedlings.

To further investigate the role of ACC deaminase, additional experiments were conducted, using Msb3ΔacdS treated plants. They significant increase in total seedling length that was observed in the Msb3-inoculated plants was completely suppressed in the Msb3ΔacdS treated plants (Figure 12). This suggests that the increase in seedling length is dependent on the presence of ACC deaminase, indicating its crucial role in this process.

Additionally, the most prominent effect of Msb3-mediated ACC hydrolysis was not in the facilitation of primary root elongation, but rather in the elongation of the hypocotyl (Figure 12). This observation indicates that the activity of Msb3 and ACC hydrolysis have a more significant impact on the elongation of the hypocotyl compared to the primary root.

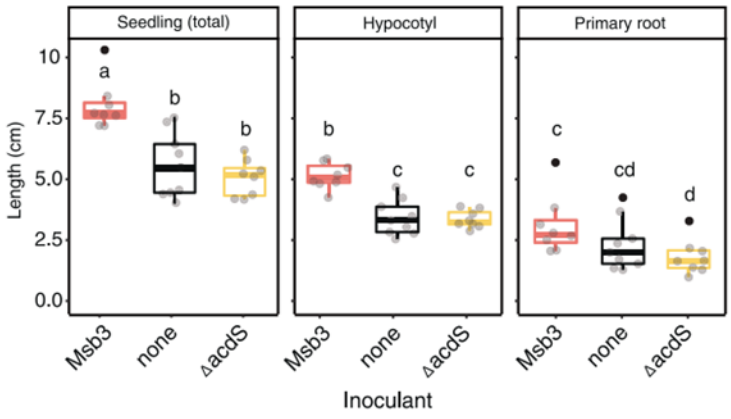

FIGURE 12 | **Phenotypic changes in tomato induced by Msb3 or Msb3ΔacdS:** Lenghts of various phenotypic parameters, taken from tomato seedlings grown in JM at 7 DPI with either Msb3, Msb3ΔacdS, or a non-inoculated control (n=8). Statistical significance was determined within each treatment and across all parameters using ANOVA, and the letters indicate the results of the Tukey post hoc test. Figure adapted from Herpell et al. (2023).

4.2.6. Impact of Msb3 and Msb3ΔacdS on tomato in the presence of native microbial community: foliar spray inoculation study

To evaluate the potential application of ACC deaminase-producing bacteria in non-sterile conditions, plant growth experiments using non-sterile soil and non-gnotobiotic tomato plants were performed. Throughout the experiment, the plants were exposed to the natural atmosphere and were watered with tap water. The experiment was started by selecting seven-day-old tomato seedlings. These seedlings were then immersed in bacterial suspensions or a control solution. Subsequently, each seedling was transferred to an individual pot for further growth.

After seven days of growth, two specific parameters were measured: hypocotyl length and seedling weight (Figure 13a, b). The obtained results aligned with our previous experiments conducted in laboratory conditions. The seedlings inoculated with Msb3 bacteria exhibited significant differences compared to those inoculated with Msb3ΔacdS or the control. Specifically, the Msb3-inoculated seedlings showed a substantial increase in hypocotyl length (Figure 13a) and weight (Figure 13b) compared to the other groups.

These findings highlight the efficacy of ACC deaminase-producing bacteria, such as Msb3, in promoting plant growth even under non-sterile and real-world conditions.

To explore different modes of inoculation, a different approach was chosen instead of immersing the seedlings in a bacterial suspension. After transplanting the seedlings, suspensions containing the wild-type strain of Msb3 or the *acdS* deficient strain were applied by spraying the shoot. To ensure that the shoot was specifically targeted and not the soil, the pots and soil were covered during the inoculation process. This enabled the focus to be directed towards the effects of the bacterial treatment on the shoot of the plants.

The same plant parameters, namely hypocotyl length and seedling weight (Figure 13a, b), were measured similar to the dip treatments. The aim was to assess whether the different mode of inoculation would yield any impact on the results. Once again, the seedlings that were inoculated with the wild-type strain of Msb3 displayed remarkable performance in comparison to the other groups. They exhibited significantly greater hypocotyl length (Figure 13a) and higher seedling weight (Figure 13b) in contrast to those subjected to the *acdS* deficient strain or those that were not inoculated.

These findings indicate that regardless of the mode of inoculation, the presence of the wild-type strain of Msb3 consistently resulted in superior plant performance. This reinforces the significance of ACC deaminase-producing bacteria in promoting plant growth and emphasizes the specific advantage of the wild-type strain over the *acdS* deficient strain and the non-inoculated plants.

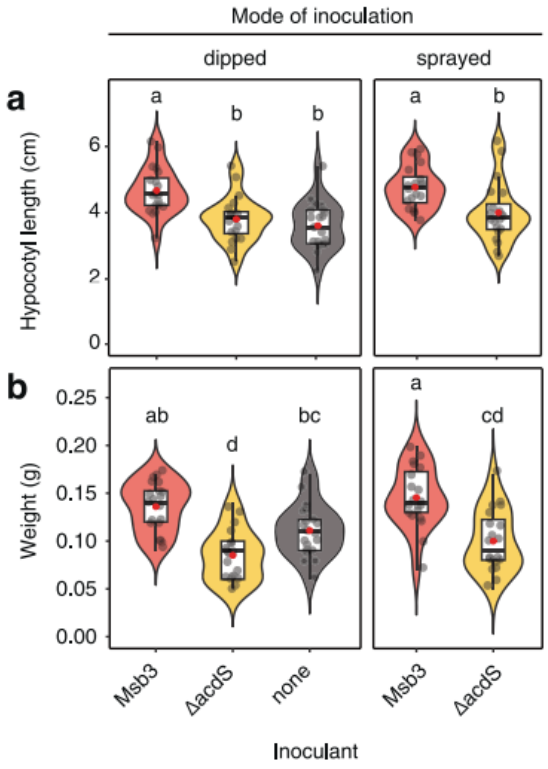

FIGURE 13 | **Results of Foliar Spray Inoculation experiment: Msb3 vs. Msb3ΔacdS: a)** Length and **b)** weight of hypocotyl of tomato seedlings cultivated in untreated soil and continuously exposed to the environment. Measurements were conducted 7DPI with either Msb3 or Msb3ΔacdS. Two modes of inoculation were compared: immersion of seedlings in a bacterial cell suspension (OD600 = 0.01) in 10 mM $MgCl_2$ during transplantation or spraying with the same suspension after transplantation. Controls were immersed in an $MgCl_2$ solution without any inoculant (n = 20 for both inoculation modes and all conditions. Statistical significance was determined within each treatment and across all parameters using ANOVA, and the letters indicate the results of the Tukey post hoc test. The mean values are indicated by red dots. Figure adapted from Herpell et al. (2023).

5. Discussion

Genetic manipulation of bacteria within the *Burkholderia* genus poses a significant challenge, primarily attributed to the lack of natural competence for DNA uptake in specific *Burkholderia* strains (Choi *et al*., 2008). It's important to mention that *Paraburkholderia*, a recently established genus that was previously classified under *Burkholderia*, also shares this challenge. The absence of this inherent ability complicates the introduction of exogenous genetic material into these organisms, impeding our ability to study and understand their functional genomics. However, despite these hurdles, recent advancements in genetic techniques have provided alternative approaches to overcome this limitation. In this study, the allelic exchange system was successfully utilized to knock out the genes of interest in

Paraburkholderia dioscoreae, strain Msb3, building upon previous studies that have demonstrated the efficacy of this system in other related species (Hamad *et al*., 2008; Wiles *et al*., 2018). By manipulating these specific genes, it became possible to evaluate their impact on promoting plant growth.

When it comes to plant growth promotion, the scientific community widely acknowledges that the hydrolysis of ACC by bacteria harboring ACC deaminase is one of the mechanisms that contribute to the promotion of plant growth, as numerous studies have demonstrated this phenomenon (Gamalero and Glick, 2015). A significant amount of research has shown that bacteria harboring the *acdS* gene or displaying ACC deaminase activity are abundant in the rhizosphere (Misra and Chauhan, 2020; Gupta and Pandey, 2019; Sarma *et al*., 2018). The evidence supporting the role of ACC deaminase in promoting plant growth comes from studies involving mutant bacteria lacking the *acdS* gene, with the focus being again on the rhizosphere (Sun *et al*., 2009). Based on the available information, prior to this study there has been no experimental validation through development of an *acdS* deficient strain demonstrating ACC hydrolysis by bacteria colonizing the phyllosphere.

Strain Msb3 is a potent phyllosphere colonizer that has an *acdS* gene and promotes plants growth. Although it can also colonize the roots to a certain extent, its abundance in the phyllosphere is tenfold higher, suggesting that a leaf-associated lifestyle is a characteristic feature of Msb3 (Herpell et al., 2020, 2023). Unlike many other bacteria commonly found on leaves (Delmotte *et al*., 2009), Msb3 is not a methylotroph. Although nitrogen fixation could potentially contribute to colonization, it was demonstrated that the presence of the *nifH* gene does not affect Msb3's ability to colonize the phyllosphere (Figure 4). In addition to methylotrophy and diazotrophy, alternative strategies to adapt to the challenging environmental conditions may be employed. These strategies involve mechanisms such as DNA and ROS (Reactive Oxygen Species)-induced damage protection through the production of enzymes like catalases and peroxidases, as well as aggregate formation facilitated by quorum sensing (Vorholt, 2012; Monier and Lindow, 2003). Along with the ability to promote plant growth, these features make Msb3 a promising candidate for use in agricultural applications, particularly in the development of biological products that can be sprayed onto leaves.

The conducted experiments demonstrated that Msb3 utilizes ACC deaminase to reduce the negative effects of ACC and promote the growth of tomato plants (Figures 11, 12, 13). Interestingly, when the *acdS* gene is absent in Msb3, its plant growth promoting (PGP) activity is significantly reduced, suggesting that this PGP process is associated with ethylene perception. The most plausible explanation for the significant influence of Msb3 inoculation of tomato plants on the length of their hypocotyls, without the presence of any inhibitory agents, is the manipulation of the ethylene pool through the tomato shoot. These effects were consistently observed in plants that were inoculated via foliar spraying, even when the soil beneath them was covered (Figure 13). These findings hold strong validity and

reproducibility, as they were observed not only in sterile lab conditions but also in the presence of a diverse microbial community (as seen in Figure 13).

Present knowledge suggests that the conserved amino acid transporters, such as LHT1, are used to transport ACC from the rhizosphere into the bacterial cytosol (Vanderstraeten, 2017). In support of this notion, previous studies demonstrated that ACC sprayed onto leaves can be taken up by Arabidopsis plants through the LHT1 transporter (Shin *et al*., 2015). The presence of a conserved ACC transport system, such as LHT1, strongly suggests the possibility of a similar mechanism for ACC transport in leaves.

The results presented in this study can be used to develop strategies for incorporating PGPB into agriculturally relevant biological products, particularly focusing on microorganisms expressing ACC deaminase. However, despite promising results obtained in controlled laboratory environments, the beneficial effects of PGPB often fail to manifest in real-world agricultural settings. Laboratory studies typically involve artificial environments and simplified systems with specific host plants and PGPB strains, which may differ significantly when exposed to diverse conditions and microbial populations in field environments (Delgado- Ramírez *et al*., 2021; Wippel *et al*., 2021). Moreover, the presence of a diverse microbial community in the soil and rhizosphere may hinder the effectiveness of specific PGPB strains, especially if they are not well-suited to compete and establish themselves within the existing microbial population (Mallon *et al*., 2015).

The phyllosphere, though, offers greater manipulation opportunities than other microbial communities (Meyer and Leveau, 2012). This accessibility is particularly significant during the early stages of plant growth but also throughout plant development (Carlström *et al*., 2019). Manipulating the microbiota during these stages, when the microbial community is still forming, has higher chances of success. The continuous accessibility of the phyllosphere during a plant's life cycle provides a valuable opportunity to introduce beneficial microorganisms. It enables interventions that can restore and maintain a balanced relationship between the host plant and its microbial community at any point during cultivation. By strategically targeting the phyllosphere, it becomes possible to optimize the use of beneficial microorganisms and maintain a healthy microbial balance for improved plant growth and development. Although promoting a natural and well-balanced microbiome highly improves agricultural productivity, this study proposes that when using a single PGPB, the most substantial benefits are obtained by applying it to the phyllosphere during the initial phases of a plant's growth.

6. Conclusion

This research contributes insights into the utilization of PGPB and ACC deaminase-expressing microorganisms in agricultural practices. It underscores the importance of understanding the specific mechanisms and dynamics involved in plant-microbe interactions, as well as the challenges associated with translating laboratory findings to real-world agricultural systems. It was done with love and hope that future studies can build upon these findings to develop innovative strategies for enhancing crop productivity and sustainability in the face of diverse microbial communities and changing environmental conditions.

*Disclaimer

The results and findings presented in this study have been previously published in The ISME Journal, with the author of this document serving as the second author. The original article can be accessed and referenced at the following URL: https://www.nature.com/articles/s41396-023-01428-7.

The provided link allows direct access to the full article and ensures accurate referencing and attribution to the original work.

Throughout this thesis, I have utilized AI tools to assist with linguistic refinement and sentence structure, while ensuring that the content and ideas remain entirely my own.

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