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Theme

A comparative genomics study of *Sinorhizobium* spp. strains highlighting the molecular mechanisms underlying partner specificity in rhizobia-legume symbiosis

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THESIS

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ABSTRACT

Bacteria from the genus *Sinorhizobium* can form a symbiotic relationship with various leguminous plants, such as the model legume *Medicago truncatula.* This plant-microbe association induces the formation of specialized plant organs called root nodules, inside which the bacteria perform symbiotic nitrogen fixation (SNF). Among the methods of controlling the symbiotic process by the plant is the secretion of phenolic compounds (PCs), which acts as signaling molecules and have a potential role in modulating bacterial communities as a defense mechanism during symbiotic nodulation. This study aims to elucidate some of the molecular mechanisms involved in the selection of plant growth promoting Rhizobacteria (PGPR) by the host and factors determining symbiont specificity. Phenolic acid bioassays coupled with a comparison of the Pan-Genome of various *Sinorhizobium* spp. strains revealed key differences in gene features and distribution, especially in strain-specific genes found predominately within one of the symbiotic megaplasmids of *S. meliloti,* pSymA. Synteny analysis showed varying presence of *rctB*, a plasmid transfer transcription regulator. The gene was not present in two organisms, *S. meliloti* AK83 and *S. medicae* WSM419; these two strains were shown to be susceptible to the antimicrobial effect of gallic acid (GA), indicating the importance of plasmid conjugation during symbiosis. Transcriptome analysis further supported this idea. We found that genes directly involved in plasmid transfer, such as *traA* relaxases and Type IV Secretion System (T4SS) proteins, genes present in pSymA, were substantially up-regulated during the N2-fixation phase of root nodulation. Protein sequence comparison showed considerable dissimilarity in proteins encoded by pSymA compared to the rest of the genome. The similar localization of plasmid transfer genes and the majority of strain-specific genes indicates the importance of the megaplasmid in determining plant-microbe compatibility and N2-fixation effectiveness. This study provides insight into the molecular differences between various strains of *Sinorhizobium* spp. influencing their symbiotic prowess and the potential basis for the control mechanisms of plant-microbe interactions.

Keywords: Rhizobia, nodules, plant phenolic compounds, bacterial conjugation, symbiosis.

List of Abbreviations

BNF	:	Biological nitrogen fixation
SNF	:	Symbiotic nitrogen fixation
PAN	:	Plant available nitrogen
PGPR	:	Plant growth-promoting Rhizobacteria
AON	:	Auto-regulation of nodulation
NF	:	Nodulation factors
LCOs	:	Lipo-chito-oligosaccharides
Rhs	:	Root hairs
ENOD	:	Early nodulin genes
PCs	:	Phenolic compounds
ROS	:	Reactive oxygen species
RNS	:	Reactive nitrogen species
BA	:	Benzoic acid
HBAs	:	Hydroxybenzoic acids
SA	:	Salicylic acid
DHBA	:	Dihydroxybenzoic acid
GA	:	Gallic acid
AFB1	:	Aflatoxin B1
HCAs	:	Hydroxycinnamic acids
DDM	:	Disc diffusion method
MHA	:	Mueller-Hinton Agar
BLAST	:	Basic Local Alignment Search Tool
NCBI	:	National Center for Biotechnology Information
PAD	:	Phenolic acid decarboxylase
MSA	:	Multiple Sequence Alignment
T4SS	:	Type IV Secretion System
HGT	:	Horizontal gene transfer

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Part I. Literature Review

Chapter 1. Rhizobia-legume symbiosis

1.1. Biological nitrogen fixation

Nitrogen (N) is a crucial element to the functioning of all living organisms [1]. It is present in many major biomolecules including amino acids, ATP, and nucleic acids. Plants are highly dependent on nitrogen for their growth and function as it constitutes a significant portion of Chlorophyll, a vital pigment for the process of photosynthesis [3]. Plants can only utilize Nitrogen in the form of inorganic Nitrate (NO_3^-) or Ammonium (NH_4^+) known as plant available nitrogen (PAN) [4, 6]. Despite its great abundance in the Earth's atmosphere as dinitrogen (N_2), Nitrogen is the key limiting factor for plants due to the constrained availability of its reduced form in soils [2]. Nitrogen can be reduced to PAN by one of four processes: the Haber-Bosch process, organic matter decomposition, the natural conversion of atmospheric nitrogen, or biological nitrogen fixation (BNF) [5, 7].

Biological Nitrogen Fixation is an important process for enhancing soil fertility and plant growth [4, 12], but it also plays a role in the biogeochemical cycle of Nitrogen; BNF is estimated to result in the creation of 200 Tg of organic N every year [13].

BNF is performed by Diazotrophs, a select group of prokaryotes which can utilize the enzyme nitrogenase to catalyze the conversion of atmospheric Dinitrogen (N_2) into more bioavailable compounds such as ammonia (NH_3) [8]. BNF requires micro-aerobic conditions for the proper functioning of the enzyme nitrogenase within N_2-fixing tissue, as well as ensuring the sufficient production of ATP for both bacterial and host cells [11, 14].

The two types of Diazotrophs are free-living, which can directly fix atmospheric nitrogen, and Symbiotic Diazotrophs, which perform symbiotic nitrogen fixation (SNF) within specialized plant organs [9,10]. Nitrogen-fixing bacteria are present in various different environments, ranging from terrestrial to aquatic habitats [14]. The most common Symbiotic Diazotrophs are aerobic Gram-negative bacteria, typically referred to as Rhizobia, a group of bacteria capable of entering a symbiotic relationship with plants from the *Leguminosae* family [10].

1.2. Legume nodulation

Legumes are a group of flowering plants in the family *Fabaceae,* previously named *Leguminosae* [47]. They are more commonly referred to as grains or pulses in the vernacular. Due to containing a high content of protein and fiber, as well as a number of significant phytochemicals, legumes make a valuable source of nutrients for humans [48]. Growing legumes also has a drastic environmental and economic implications, as the majority of legumes enter a symbiotic relationship with Nitrogen fixing bacteria, resulting in the fixation of an approximate 200 million tonnes of atmospheric N2 annually; this greatly improves soil denitrification and reduces the need for expensive synthetic fertilizers [49, 50].

Legumes evolved the ability to host plant growth-promoting Rhizobacteria (PGPR) within specialized organs (nodules) around 58 million years ago [51]. This symbiotic association occurs in 88% of legume species [52].

The tissue of root nodules is ideally suitable for the process of SNF carried out by the bacteria. Inside plants, bacteria differentiate into specialized N2-fixing forms called bacteroids [57]. In this mutually beneficial relation, the bacteria provide the plant with bioavailable nitrogen essential for plant activity in exchange for acquiring carbohydrates derived from photosynthesis [55].

1.2.1 Nodule organogenesis

The nodulation process of the rhizobia-legume symbiosis is initiated by the plant, which secrete flavonoid molecules whose function is attracting bacterial partners to the plant's roots [53]. The recognition of these compounds by compatible bacteria triggers a specific signaling cascade between the plant and bacteria, leading to the formation of the root nodules [53, 54]. A key element of the successful induction of plant nodules is the coordinated regulation of various genes involved in the molecular signaling between the plant and bacteria [58]. The main regulation pathway is the auto-regulation of nodoulation (AON) pathway [59].

Nodule organogenesis is a multi-stage process: (1) flavonoid secretion and root hair deformation; (2) cortex cell differentiation; (3) development of a transient structure, the nodule primordium; (4) bacterial growth and invasion through the infection thread (IT); (5) bacteroid differentiation; (6) nodule maturation and beginning of N2 fixation; (7) senescence [53, 56].

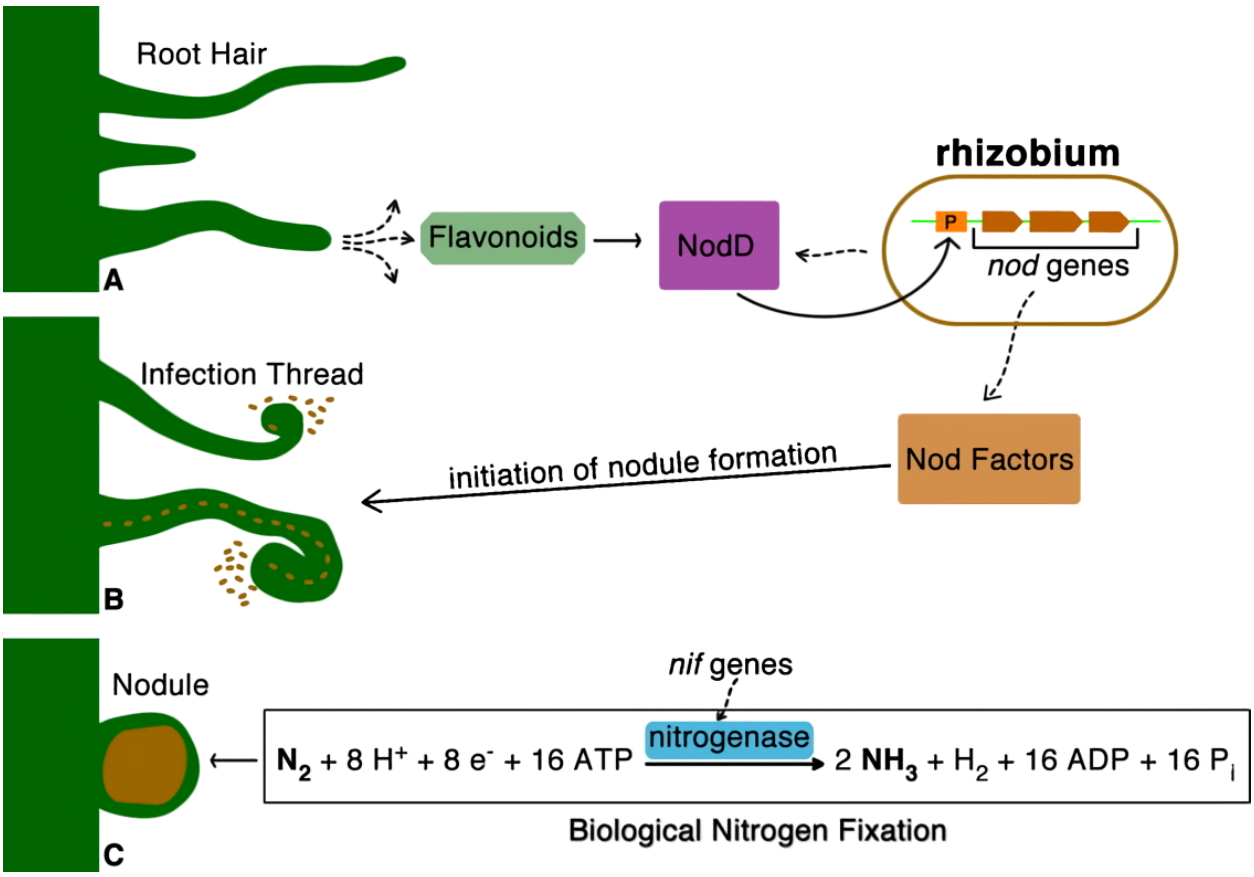


Figure 1. Schematic overview of the major steps of plant nodule formation and biological nitrogen fixation (BNF). [65]

1.2.1.1 Nod factors

Nodulation factors (NF) are bacterial strain-specific lipo-chito-oligosaccharides (LCOs) stimulated by plant flavonoids as part of the AON pathway and act as signal molecules [59]. NFs elicit morphological changes in the host plant, such as the emergence and deformation of plant root hairs (RHs) and nodule primordia, essential structures for the invasion of bacteria into plant cells [60]. NFs have an impact on plant hormones and mitotic activities of host cells [61]. NFs additionally have an effect on tissue-specific transcription levels of early nodulin genes (ENOD) which have a role the early steps in root nodule formation [63].

Nodulation factors are synthesized by enzymes encoded by bacterial *nod* genes [62, 64]. The gene *nodD* encodes a transcriptional activator upstream of the *nod* gene operons, called the *nod* box; it activates the expression of other *nod* when detecting root exudates [64].

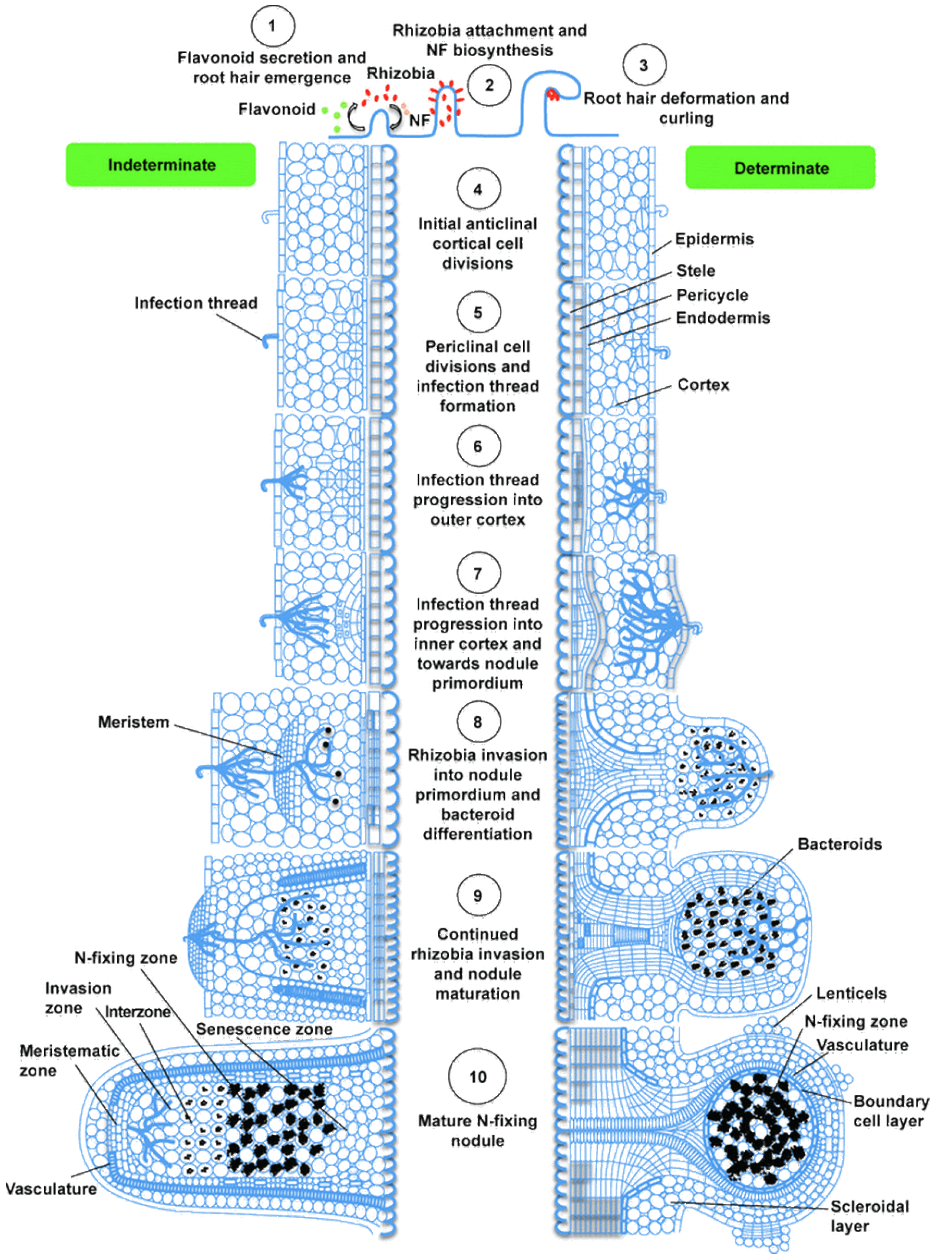


Figure 2. Stages of nodule organogenesis in both determinate and indeterminate nodule types [53].

1.2.2 Nodule anatomy

1.2.1.1 Determinate and indeterminate nodules

Root nodules can be classified into two types, determinate and indeterminate nodules depending on their morphological characteristic following nodulation [66]. Indeterminate nodules, such as those in *Medicago truncatula, Pisum sativum, Vicia faba, and Trifolium repens*, have a

persistent, functional meristem which allows for the continued growth of the nodule, whereas determinate nodules have non-persistent meristems and in which all the infected cells are at a similar stage of development (e.g. the nodules of *Glycine max and Lotus japonicus)* [67].

1.2.1.1 Zonation of indeterminate nodules

Indeterminate nodules have a very different structure to determinate nodules, being segmented into five clearly distinct zones, each acting as its own ecological niche [68]. The five zones are [69]:

- Zone I: The active meristem allowing the growth of the nodule.
- Zone II: The infection zone where the symbiosome housing differentiated bacteroids is formed.
- Zone III: The Nitrogen fixation zone; bacteria are most proficient at capturing atmospheric N.
- Zone IV: The senescence zone; plant and bacterial cells are deconstructed and symbiosis is diminished.
- Zone V: The free-living zone; bacteria return to a non-differentiated state and the structure of the symbiosome is lost.

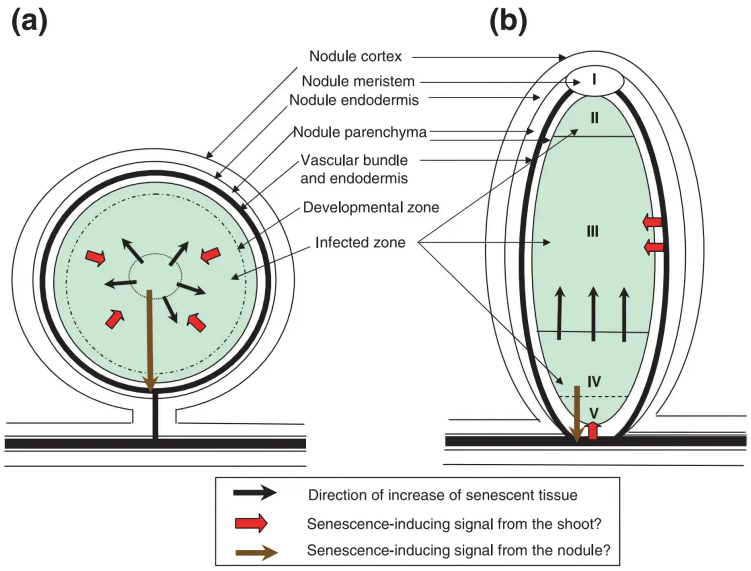


Figure 3. Diagram representing the different structure of determinate (a) and indeterminate (b) nodules [69].

1.3. Rhizobia

Rhizobia is an umbrella term referring to a group of nitrogen-fixing bacteria present predominately in soils. They are gram negative, motile rod shaped bacteria; their typifying characteristic is their ability to establish a symbiotic relationship with leguminous plants and induce the formation of root nodules [15]. Following plant infection, rhizobia can perform SNF inside the resulting root nodules. Rhizobia cannot fix N2 in the free-living state and require the host-plant to do so [70]. Rhizobia exhibit elevated, translucent, viscid colonies when grown on Yeast Mannitol Agar media [71]. Rhizobia-legume symbiosis is the highest yielding BNF interaction in terms of the resulting quantity of bioavailable nitrogen [55].

The host-microbe interaction between rhizobia and legumes can be species specific, wherein only certain species of plants and bacteria can successfully establish a mutually beneficial relationship [72]. This specificity can arise from many possible mechanisms. A major determinant of host-specificity was shown to to be the variability in the common *nod* genes required for symbiosis [73]. The *nif* genes, which are highly important protein-coding genes encoding the sub-units of the enzyme nitrogenase [14]; together with *nod* genes they are one of the most studied genes involved in the rhizobia-legume symbiosis, and potentially confer a degree of partner specificity between the plants and bacteria [74].

1.3.1 Taxonomic diversity

Rhizobia are a large and diverse group, spanning the two classes of alpha- and betaproteobacteria. The majority of rhizobia are part of the *Hyphomicrobiales* order of alpha-proteobacteria [75]. Currently, rhizobia consist of over 180 species classified into 21 genera, with the most dominant being: *Rhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium, and Devosia* [76]. The polyphyletic nature of the rhizobia group entails continued refinement and more modern classification methodology.

1.3.1.1 Sinorhizobium branch

The genus *Sinorhizobium*, known synonymously as *Ensifer*, was first described in 1988 [77] and later combined with an earlier described genus into a single taxon [78]. The term *Sinorhizobium* is combination of the Latin *sino* (“China”) with the Greek *rhiza* (“root”) and *bium* (“life”) [77].

The genus is comprised of 17 species, with multiple having whole-genome sequences (e.g. *S. meliloti, S. medicae,* and *S. fredii*) [79, 80].

Table 1 Classification of six Rhizobia genera and their Host plant species

Genus	Host Plant	References
Rhizobium	Pisum Lathyrus, Vicia, Lens, Phaseolus, Trifolium Lupinus, Ornithopus Phaseolus vulgaris, Desmodium, Stylosanthes, Centrosema, Tephrosia, Acacia, Zornia, Macroptilium, Medicago ruthenica, Sesbania herbacea, Coronilla, Gueldenstaedtia, Amphicarpaea, Hedysarum coronarium, Indigofera, Astragalus	[16–22]
Mesorhizobium	*Lotus, Lupinus, Anthyllis, Leucaena Astragalus, Cicer arietinum, Glycyrrhiza, Sophora Caragana, Halimodendron, Swainsonia, Glycine, Acacia, Prosopis, Chamaecrista, Amorpha fruticosa*, *Prosopis, Astragalus adsurgens*	[19, 22–36]
Allorhizobium	Neptunia natans	[46]
Bradyrhizobium	*Glycine max, Glycine soja, Vigna, Lupinus Mimosa, Acaci*	[35–39]
Sinorhizobium	*Melilotus, Medicago, Trigonella Glycine, Cajanus, Glycine, Sesbania, Acacia, Sesbania, Prosopis, Prosopis, Kummerowia stipulacea, Leucaena leucocephala*	[40–45]
Azorhizobium	Sesbania rostrata	[31]

Chapter 2: Phenolic compounds

Poloyphenols or phenolic compounds (PCs) are a large and diverse group of plant secondary metabolites. These bioactive molecules have significant biological functions and are widespread in nature [82]. PCs are compounds which contain at least one phenol moiety consisting of a benzene ring (−C6H6) bonded to a hydroxyl group (-OH) with the general formula: C6H5OH [83]. PCs are ubiquitous in the plant kingdom; they are present in various types of fruit and vegetables (e.g. apple, banana, orange, onions, cabbage, broccoli, etc.) as well as other types of plant tissue (e.g. roots, leaves, nuts, etc. [84]. Dietary PCs have numerous health benefits to humans, including reducing the risk of metabolic disfunction and cancer-prevention, acting as natural antioxidants, improving longevity and gut health, etc. [85].

Plant polyphenols have been extensively studied largely for their antioxidant capacity to scavenge free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), reducing the harmful effects resulting from the over-accumulation of such molecules [86].

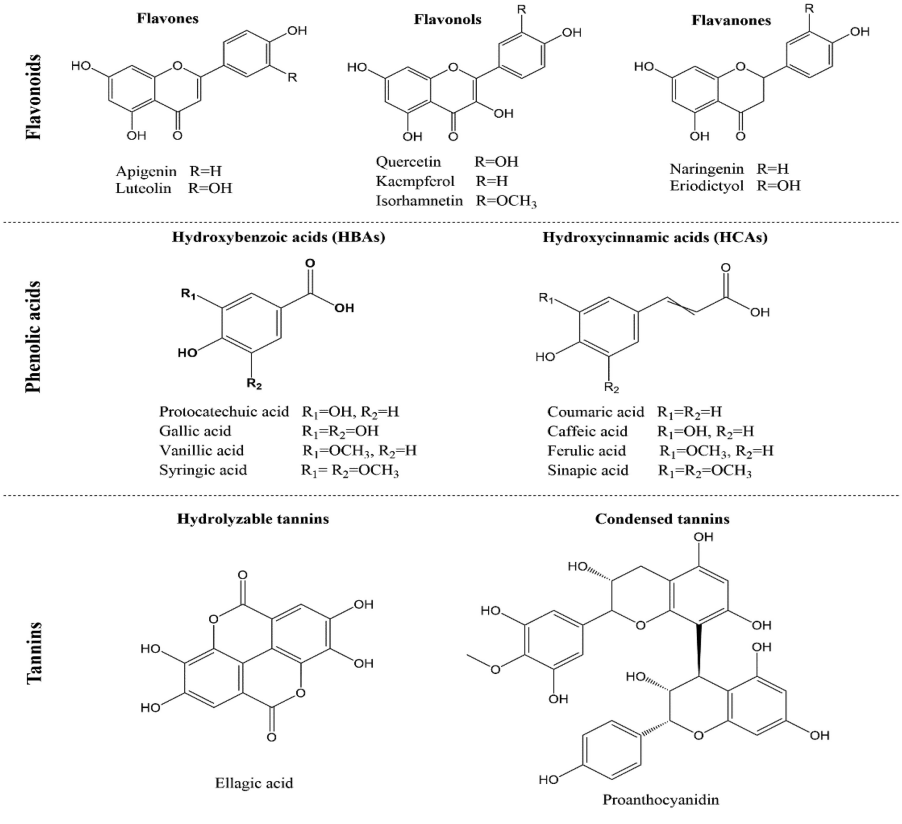


Figure 4. The chemical structure of phenolic compounds found in plants [81].

2.1 Classification of phenolic compounds

Phenolic compounds form a diverse group, which are typically categorized on the basis of skeletal carbon atoms: C-6 (simple phenols, benzoquinones), C6-C1 (phenolic acids, aldehydes), C6-C2 (acetophenones, phenylacetic acids), C6-C3 (hydroxycinnamic acids, coumarins, chromones, phenylpropanes etc.), C6-C4 (naphthoquinones), C6-C3-C6 (flavonoids, isoflavonoids, neoflavonoids, etc.), (C6-C3)2 (lignans, neolignans, oxyneolignans), (C6-C3)n (lignins), (C6-C3-C6)n (condensed tannins), etc. [87].

Table 2. Classification of Phenolic compounds based on the number of carbon atoms [88]

Number of C atoms	***Basic skeleton***	***Class***
6	C_6	Simple phenols, benzoquinones
7	C_6–C_1	Phenolic acids
8	C_6–C	Acetophenone, phenylacetic acid
9	C_6–C_3	Hydroxycinnamic acid, polypropene, coumarin, isocoumarin
10	C_6–C_4	Naphthoquinone
13	C_6–C_1–C_6	Xanthone
14	C_6–C_2–C_6	Stilbene, anthrachinone
15	C_6–C_3–C_6	Flavonoids, isoflavonoids
18	$(C_6$–$C_3)_2$	Lignans, neolignans
30	$(C_6$–C_3–$C_6)_2$	Biflavonoids
n	$(C_6$–$C_3)n$	Lignins
	$(C_6)n$	Catecholmelanine
	$(C_6$–C_3–$C_6)n$	Condensed tannins

2.1.1 Flavonoids

Flavonoids are a class of polyphenolic molecules with variable phenolic structures, each containing a 15-carbon skeleton and two aromatic rings [88]. Flavonoids are very prevalent in plants, present in all types of plants [89]. They have important roles in various plant functions, such as pigmentation, pollination, UV filtering, signaling and regulation, etc.[89, 91]. Furthermore, flavonoids are essential for plant growth and self-defense [92]. They are also implicated plant-microbe signaling [52].

Among PCs, flavonoids are especially noteworthy for their potent antioxidant and antiinflamatory potency, likely conferred by the presence of the free hydroxyl group on the third carbon atom [94, 95].

2.1.2 Tannins

Tannins are natural polyphenolic compounds with polyhydroxyphenolic groups or their derivatives attached to an aromatic ring [96]. They are characterized by their strong affinity for forming complexes with different minerals and macromolecules such as proteins and polysaccharides [97]. Due to their high polyphenic content, tannins also possess remarkable antioxidant properties; they effectively scavenge ROS and reduce oxidative stress, as well as inhibit lipid peroxidation [98]. Tannins are also known for their antimicrobial capabilities, such as disrupting bacterial cell walls and enzyme inhibition [99].

2.1.3 Phenolic acids

Phenolic acids are the simplest of the polyphenols in terms of their chemical formula. They are characterized by having one or more hydroxyl groups attached to a benzene ring [100]. Phenolic acids are widely distributed within the plant kingdom, being present in fruits, vegetables, nuts, and seeds. Like other phenolic compounds, phenolic acids have a number of diverse biological activities, chief among them is their protective properties against ROS and RNS induced damage [100, 102].

Phenolic acids can also act as signaling molecules in plant-microbe interactions [104], such as in the initiation phase of the rhizobia-legume symbiosis, showing spatially and temporally regulated expression during nodulation [105].

Phenolic acids are divided into two subgroups: hydroxycinnamic acids (C3–C6) and hydroxybenzoic acids (C1–C6) [101].

2.1.3.1 Hydroxybenzoic acids

Hydroxybenzoic acids (HBAs) include a C_6-C_1 backbone derived from benzoic acid (BA) [106]. Examples include: e.g., salicylic acid (SA), 2,5-dihydroxybenzoic acid (2,5-DHBA), 3,4-dihydroxybenzoic acid (3,4 DHBA), 2,3-dihydroxybenzoic acid (2,3-DHBA), gallic acid (GA), and vanillic acid. The structural differences in HBAs is due to aromatic ring hydroxylation and methylation [107].

2.1.3.1.1 Gallic acid

Gallic acid (GA), also known as 3,4,5-trihydroxybenzoic acid, is a polyphenolic trihydroxybenzoic acid with the general formula $C_6H_2(OH)_3CO_2H$ [109]. Gallic acid has a number of remedial effects on cardiovascular diseases, neurological diseases, diabetes, liver fibrosis, and tumors, strictly due to its anti-inflammatory and antioxidant activities [110]. Given its polyphenolic nature, GA possesses a strong capacity to scavenge ROS, and even attenuate Aflatoxin B1 (AFB1) toxicity [111]. It provides new interesting prospects for the treatment of such diseases.

GA is also known to have potent bactericidal properties against many gram negative bacteria [112]. Its antimicrobial activity is purported to result from the ability to alter membrane structures and hinder bacterial metabolism GA can also significantly alter surface hydrophobicity, charge, and K+ leakage [114]. Given its affinity to ROS, GA can also induce cellular apoptosis by targeting specific signaling pathways [113].

2.1.3.2 Hydroxycinnamic acids

Hydroxycinnamic acids (HCAs) or hydorxymates contain a C_6-C_3 backbone consisting of a benzene ring attached to a three-carbon propyl chain [106]. HCAs occur in several conjugated forms, including amide-conjugated forms (peptides, and mono or polyamines) and esters (sugar derivatives and glycosides). Examples include: caffeic acid, ferulic acid, p-coumaric acid, and sinapic acid [108].

Introduction

The rhizobia-legume symbiosis is one of the most impactful associations between biological organisms. The increase in bioavailable nitrogen results in greatly improved soil fertility and plant growth [2, 5]; this has a greatly beneficial effect on agriculture yield, providing a higher quality source of nutrients (especially protein and fiber) [48], as well as resulting in more stable and diverse terrestrial ecosystems [12, 79].

As sessile organisms, plants interact with the rest of the environment mainly through chemical signaling. For plants to enter a mutually beneficial relationship with Rhizobia bacteria, a degree of compatibility is required [52, 54]. Both plants and bacteria employ various mechanisms to ensure the selection of the most suitable symbiont. One of the strategies used by plants for partner selection is the secretion of various secondary metabolites known as phenolic compounds [89, 94, 105] that modulate the surrounding microbial community and guide compatible bacteria during the many phases of the symbiotic relationship.

The goal of this study is to help elucidate the molecular mechanisms underlying the intricate symbiont specificity in rhizobia-legume associations at all stages of the process. Understanding the factors governing bacterial infection and survival inside host-plants, as well as the effectiveness of the resulting BNF could lead to major breakthroughs in ecosystem engineering and agriculture.

Research Methodology:

1. In vitro tests of plant phenolic acids against select strains of Rhizobia bacteria
2. Synteny analysis of the studied strains highlighting relevant genes
3. Genome analysis investigating the cloud genome
4. Functional annotation of symbiotic megaplasmids
5. Transcriptome data analysis of different gene families during plant nodulation
6. Phylogenetic tree construction and evaluation of evolutionary relatedness
7. Protein-sequence based comparison using bidirectional BLASTP

Part II. Materials and Methods

1. Strains used in the study

The various strains of *Sinorhizobium* spp. that were used in this study are listed in Table 3. These strains were specifically selected for possessing differing BNF prowess and unique plant host-compatibility within symbiotic relationships [122].

Table 3. Strains used in the study

Strain	Source/Reference
Sinorhizobium meliloti 2011	[115]
Sinorhizobium meliloti AK83	[116]
Sinorhizobium meliloti SM11	[117]
Sinorhizobium meliloti CCMM B554 (FSM-MA)	[118]
Sinorhizobium meliloti 1021	[119]
Sinorhizobium meliloti WSM1022	[120]
Sinorhizobium medicae WSM419	[121]

2. Gallic acid antimicrobial bioassay

Susceptibility to GA was determined using the Kirby-Bauer test, also known as the disc diffusion method (DDM), on various *Sinorhizobium* spp. strains. The test was performed using a solution of 0.01 g/ml of GA. A volume of 10 µl was added to sterile filter paper discs on Mueller-Hinton Agar (MHA) plates. The plates were incubated at 30℃ for 48h. Sterile distilled water was used as a negative control disc.

3. Genome Comparison

3.1 BLAST Search

Using the Basic Local Alignment Search Tool (BLAST) [123] provided by the National Center for Biotechnology Information (NCBI), the gene encoding a phenolic acid decarboxylase (PAD) enzyme involved in gallic acid degradation, annotated as UbiD-familly gallate decarboxylase [124], was used as query and compared against the order *Hyphomicrobiales*.

3.2 Synteny analysis

Genome sequence and annotation of the studied *Sinorhizobium* strains [Table 3.] were downloaded from the NCBI Reference Sequence Database (RefSeq). Synteny analysis was performed using the One Step MCScanX and Multiple Synteny Analysis features available on the Toolbox for Biologists (TBtools) computer software.

3.3 Pan-Genome analysis

The Pan and Core-Genome analysis was performed and visualized using the Comparative Genomics toolset available on MicroScope, an online Microbial Genome Annotation & Analysis Platform. Analysis of the Core-Genome distribution, accessory genes, and strain-specific genes was done using Pan-genome explorer.

Functional annotation of the two symbiotic megaplasmids of *Sinorhizobium* was achieved using the RAST tool kit (RASTtk) and visualized using the Comparative Systems tool provided on the Bacterial and Viral Bioinformatics Resource Center (BV-BRC).

4. Phylogenetic analysis

To better understand the evolutionary relations between the studied organisms, a Phylogenetic tree was constructed using the 16s rRNA genes of closely related Rhizobia bacteria. The Molecular Evolutionary Genetics Analysis (MEGA) software version 11 [127] was utilized to perform the Multiple Sequence Alignment (MSA) and phylogenetic tree construction. The MUSCLE method [128] was used for MSA and the Maximum Likelihood model [129] and the bootstrap method [130] (100 replications) for Phylogenetic tree construction and testing. *Bacillus subtilis* was chosen as the outgroup. All data was downloaded from the NCBI Reference Sequence Database (RefSeq).

5. RNA-seq data analysis

The transcriptome data for specific nodule zones during rhizobia-legume symbiosis was obtained using RNA sequencing and microdissection of the nodule regions of *Medicago truncatula* [125] and was then downloaded from the dedicated website. The data was plotted and customized using Microsoft Excel [126].

6. Proteome comparison

Proteome data was downloaded from the Universal Protein Resource (UniProt) reference proteome database. Comparison and visualization was carried out using the bidirectional BLASTP algorithm [123] available in the Proteome Comparison Service on the Bacterial and Viral Bioinformatics Resource Center (BV-BRC). Six *Sinorhizobium* spp. strains' proteomes were compared against that of the reference genome of S. meliloti strain MABNR56.

Part III. Results

1. Strain-dependent susceptibility of *Sinorhizobium* spp. to gallic acid

The results of disc diffusion test showcase differing susceptibility to GA by the different strains tested. With the exception of *S. meliloti* AK83 and *S. medicae* WSM419, whose growth was inhibited, the majority of the strains appear to be resistant to the antimicrobial effects of GA.

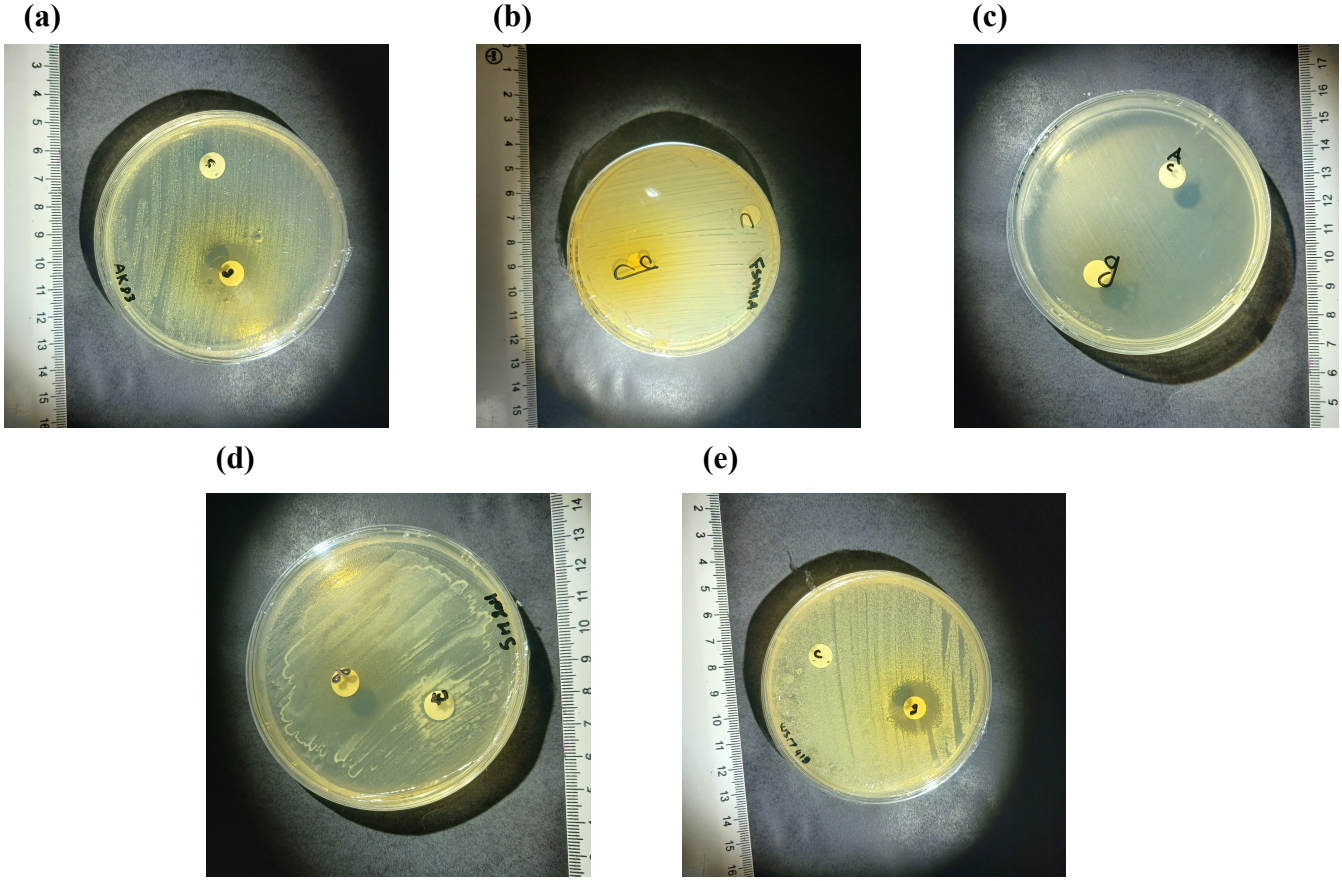


Figure 5. Results of the gallic acid antimicrobial bioassay using the disc diffusion method. Strains tested: (a) *S. meliloti* AK83 b) *S. meliloti* CCMM B554 (FSM-MA) (c) *S. meliloti* SM11 (d) *S. meliloti* 1021 (d) *S. medicae* WSM419. Strains CCMM B554, SM11, and 1021 exhibit normal growth, indicating resistance to GA. The inhibition ring around the cultures of AK83 and WSM419 is an indication to GA susceptibility.

2. Varying localization of phenolic compound decarboxylase and plasmid transfer genes

Basic Local Alignment Search (BLAST) results showed different localization of the gene coding for gallate decarboxylase across multiple species of *Rhizobium*, *Agrobacterium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, etc.

Synteny analysis revealed high gene conservation of gallate decarboxylase in Symbiotic plasmid B (pSymB) of *Sinorhizobium* spp, a highly syntenic chromosome-like megaplasmid [131], while *rctA* and *rctB*, genes involved in regulating plasmid conjugation and transfer encoded by pSymA [132],display relatively poor conservation across the analyzed strains.

Table 4. Location of UbiD Family decarboxylase across various genera

Genus	Species	Location
Sinorhizobium	*meliloti*	Plasmid
	medicae	Plasmid
	fredii	Chromosome
Agrobacterium	*salinitolerans*	Plasmid
	tumefaciens	Plasmid
	pusense	Chromosome
	fabrum	Chromosome
Mesorhizobium	*australicum*	Chromosome
	sp. AR07	Chromosome
	sp. NZP2298	Chromosome
	sp. 131-3-5	Plasmid
Rhizobium	*gallicum*	Plasmid
	sullae	Plasmid
	rhizogenes	Plasmid
Bradyrhizobium	*oligotrophicum*	Chromosome
	ontarionense	Chromosome
Azospirillum	*Brasilense*	Chromosome
	lipoferum	Chromosome
	ramasamyi	Chromosome

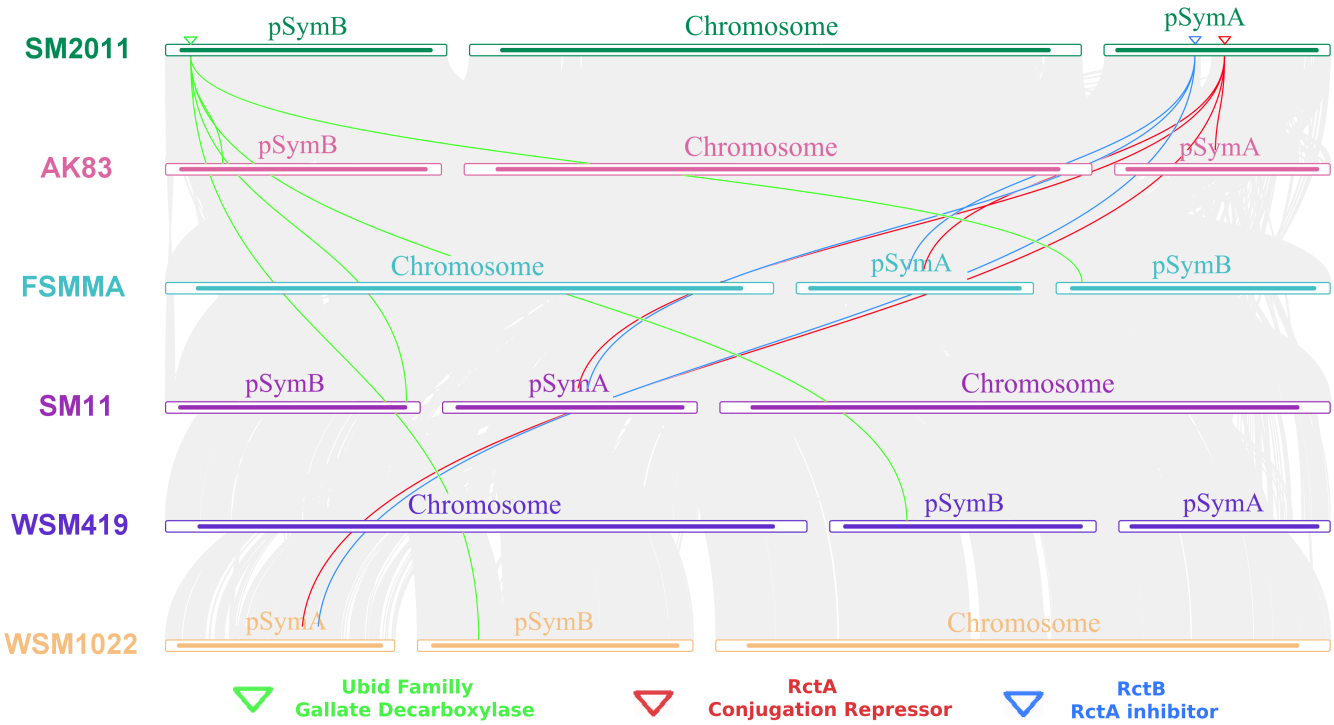
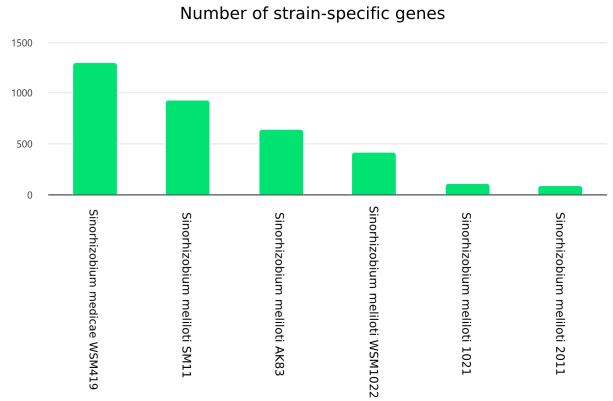


Figure 6. Synteny Plot highlighting the positions of relevant genes. The graph was generated using the Multiple Synteny Analysis feature in TBtools. The *ubiD* gene found in pSymB is present in all tested strains, suggesting high conservation. r*ctA,* a transcriptional repressor found in pSymA which regulates plasmid conjugation is also present in all strains. *rctB*, on the other hand, the antirepressor to *rctA* is not present in *S. meliloti* AK83 and *S. medicae* WSM419.

3. Significant genome distribution and unequal count of strain-specific genes

To investigate the genetic variability underlying the difference in GA resistance, a Pan-genome analysis of the studied strains was performed. We looked at the distribution and gene count of core vs strain-specific genes.



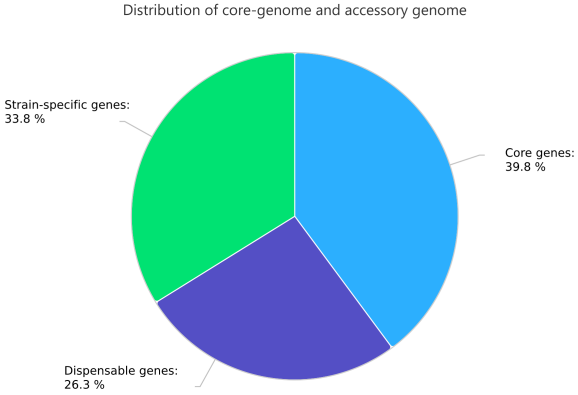


Figure 7. Pan-genome analysis highlighting strain-specific gene count. The analysis reveals a significant percentage of the pan-genome as strain specific (33.8%) with a substantial percentage composed of dispensable or accessory genes (26.3%). The strains with the highest number of strain-specific genes are showcased on the left. Strains WSM419, SM11, AK83, and WSM1022 have a considerably higher number of strain-specific genes.

4. Localization and distribution of the cloud genome

To facilitate understanding the relatedness of the studied organisms and the differences in gene content and features between them, data of the core-genome overlap and accessory genes intersection was visualized. The localization of strain-specific genes was also demonstrated.

a) b)

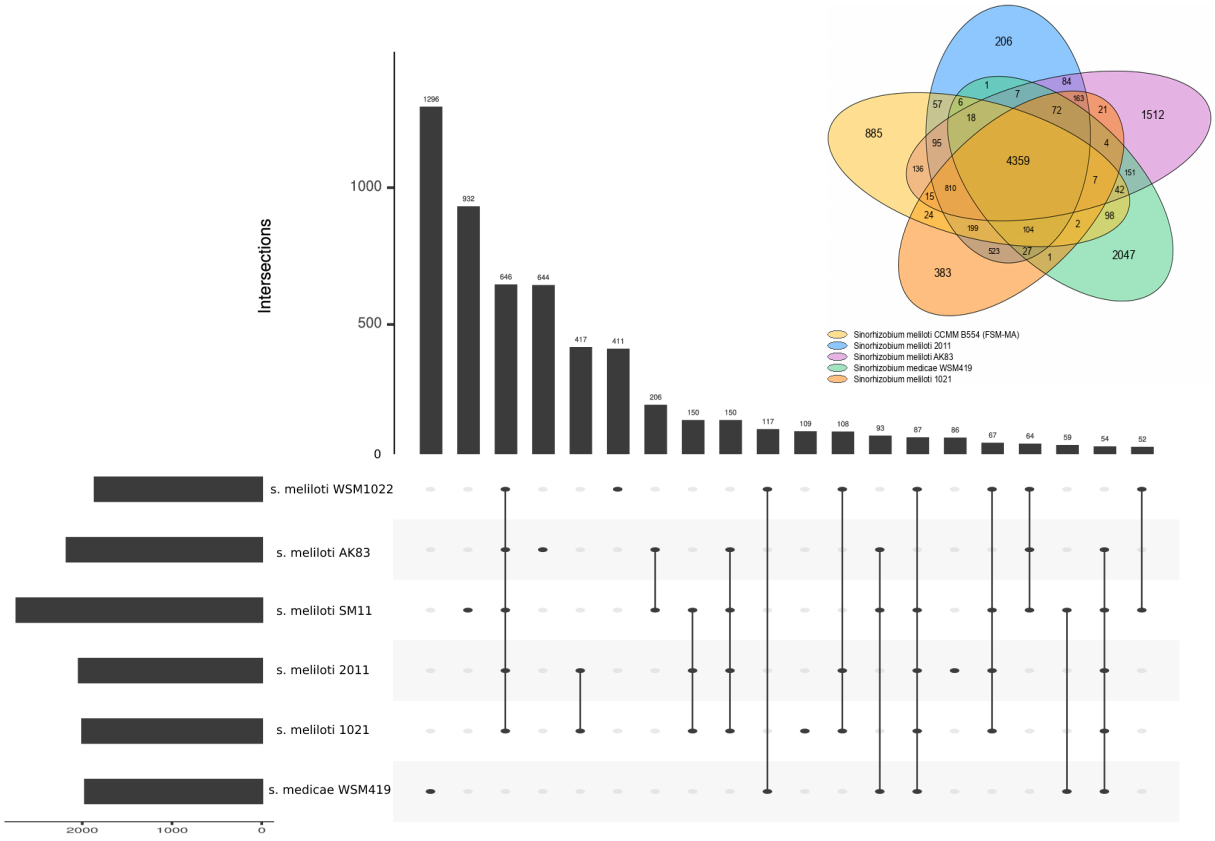


Figure 8. Diagrams depicting the core and accessory genomes. A) Upset plot of the accessory genome size and intersection of the studied strains. b) Venn diagram of the pan and core genome.

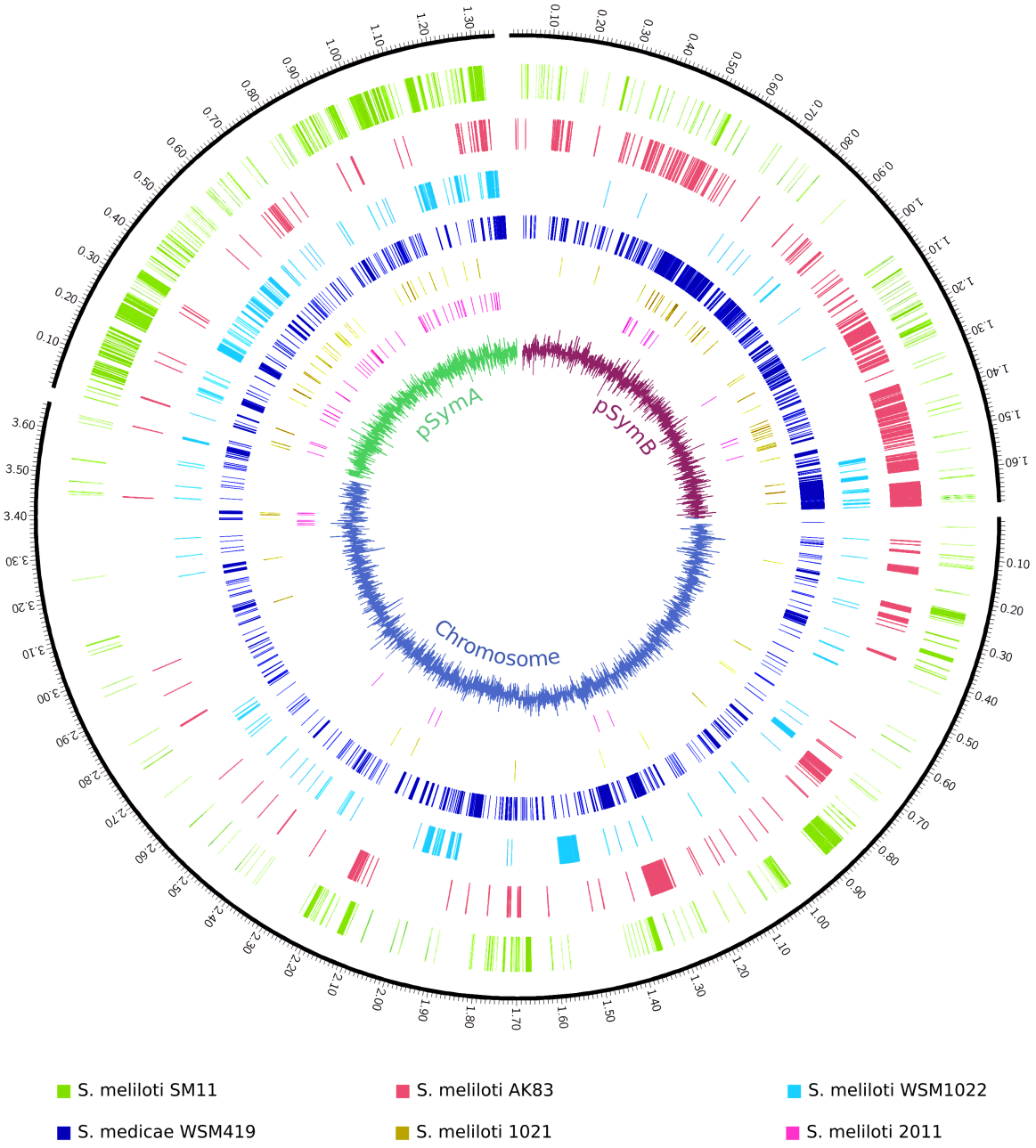


Figure 9. Circular diagram showing the localization of strain-specfic genes. Comparison of the number and positioning of strain-specific genes, i.e. genes not part of the core-genome and found only in a specific strain. *S. meliloti* spp. strains possess relatively few strain-specific genes on the chromosome compared to those found on pSymA and pSymB.

5. Disparity in gene function of symbiotic plasmids between strains

Functional annotation of the pSymA and pSymB sequences of select strains (WSM419, WSM1022, and SM11) was performed and visualized using the Comparative Systems tool of the Bacterial and Viral Bioinformatics Resource Center (BV-BRC).

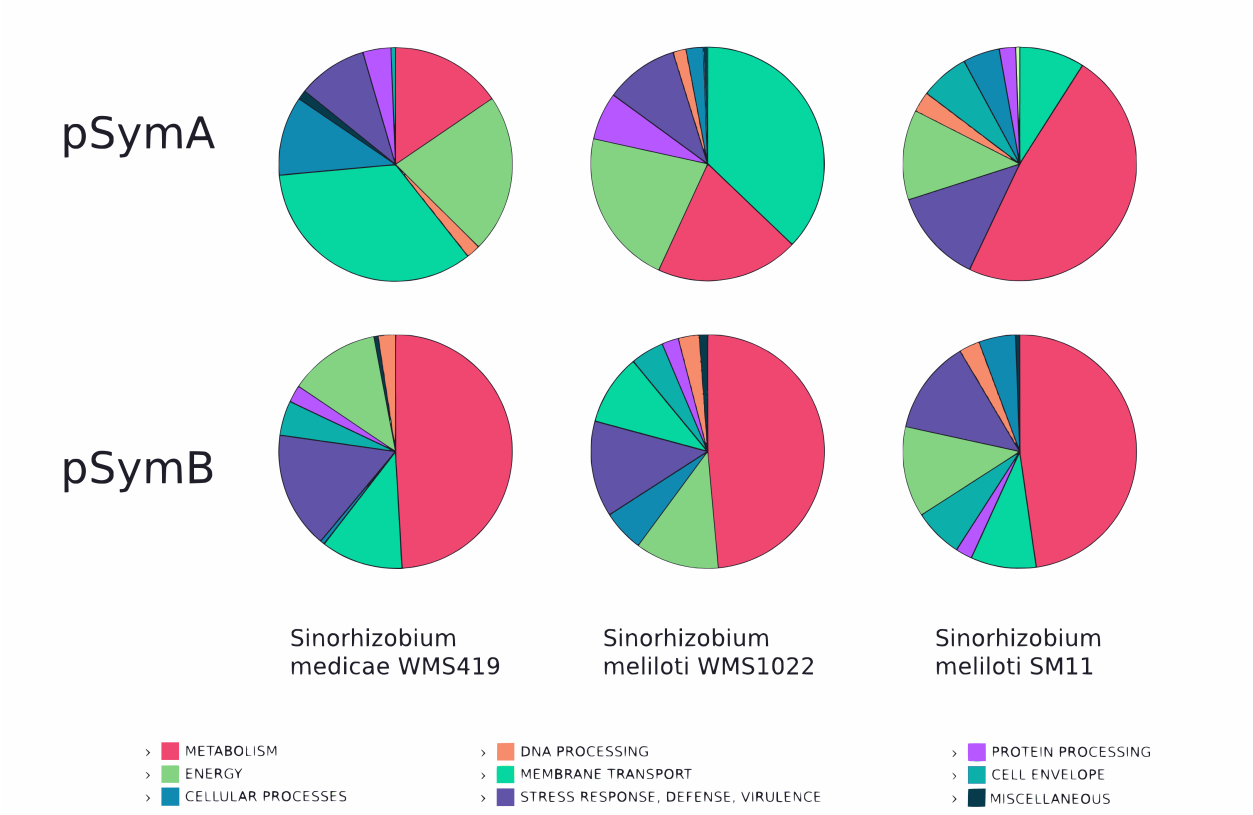


Figure 10. Pie charts featuring the functional annotation of the symbiotic megaplasmids of three *Sinorhizobium* bacteria. pSymA of *S. medicae* WSM419 and *S. meliloti* WSM1022 possesses genes responsible for various functions, with genes involved in energy production and conservation being the most abundant. In contrast, pSymA of *S. meliloti* SM11exhibits a functional distribution more similar to the chromosome, with genes involved in metabolism being more predominant. pSymB in all three bacteria shows chromosome-like distribution of gene features.

6. Phylogeny reveals distinct lineages of *Sinorhizobium* spp. strains

To gain insight into the evolutionary history and closeness of the studied strains, the 16s rRNA gene of various closely related Rhizobia bacteria was used to construct a phylogenetic tree.

Visualization of the newick tree was done by the tree viewer tool on the Environment for Tree Exploration toolkit website (ETE Toolkit).

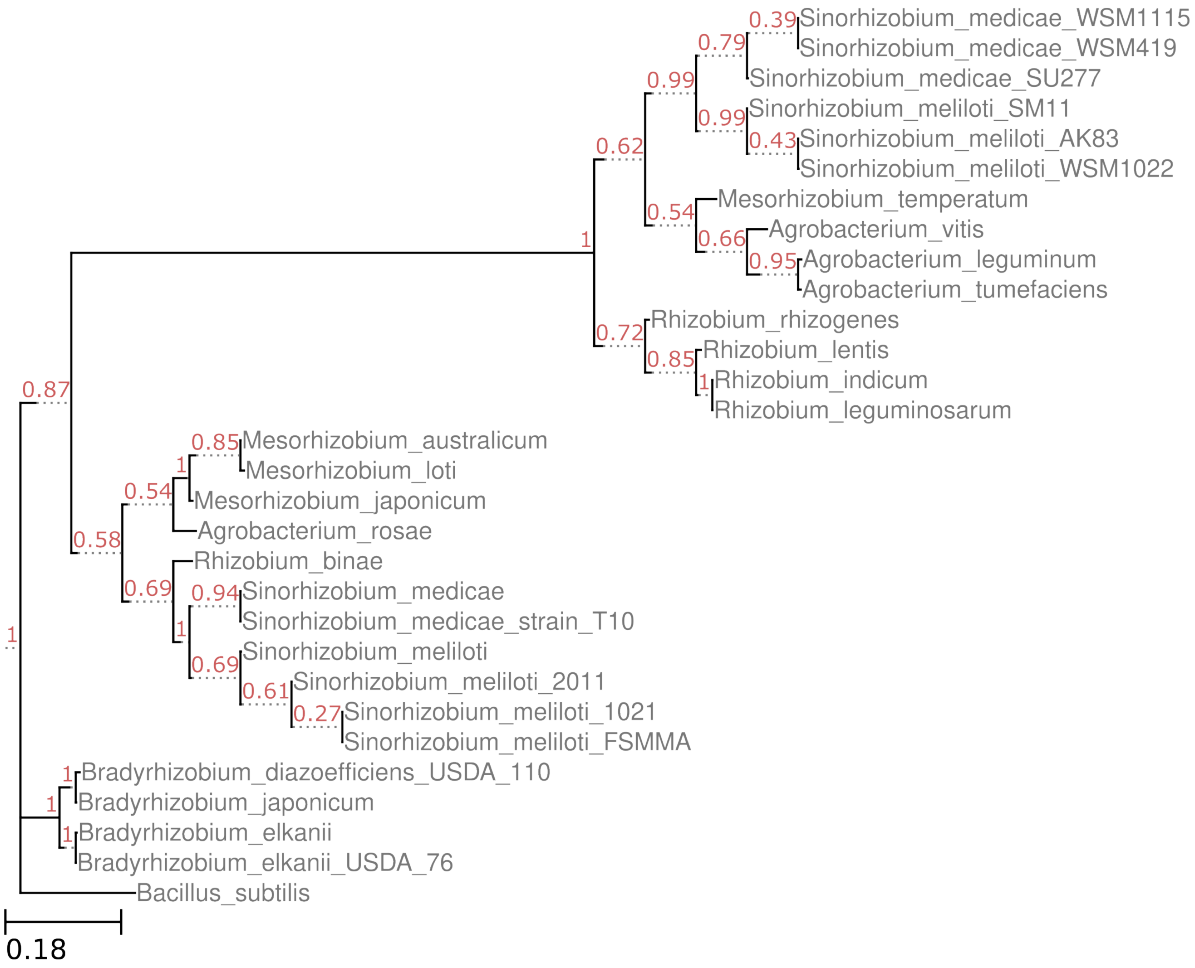


Figure 11. Phylogenetic tree constructed using the 16s rRNA gene. The tree branches into three main lineages. The earlier *Bradyrhizobium* spp. branch, and two other later lineages on which various *Sinorhizobium* spp. strains are positioned. Bootstrap test values are displayed in red.

7. Different expression levels of phenolic acid metabolism and plasmid conjugation genes within the four nodule zones

To assess the role and mechanisms of the studied genes in the context of rhizobia-legume symbiosis, *S. meliloti* RNA transcript data at different stages of nodule infection was plotted and analyzed.

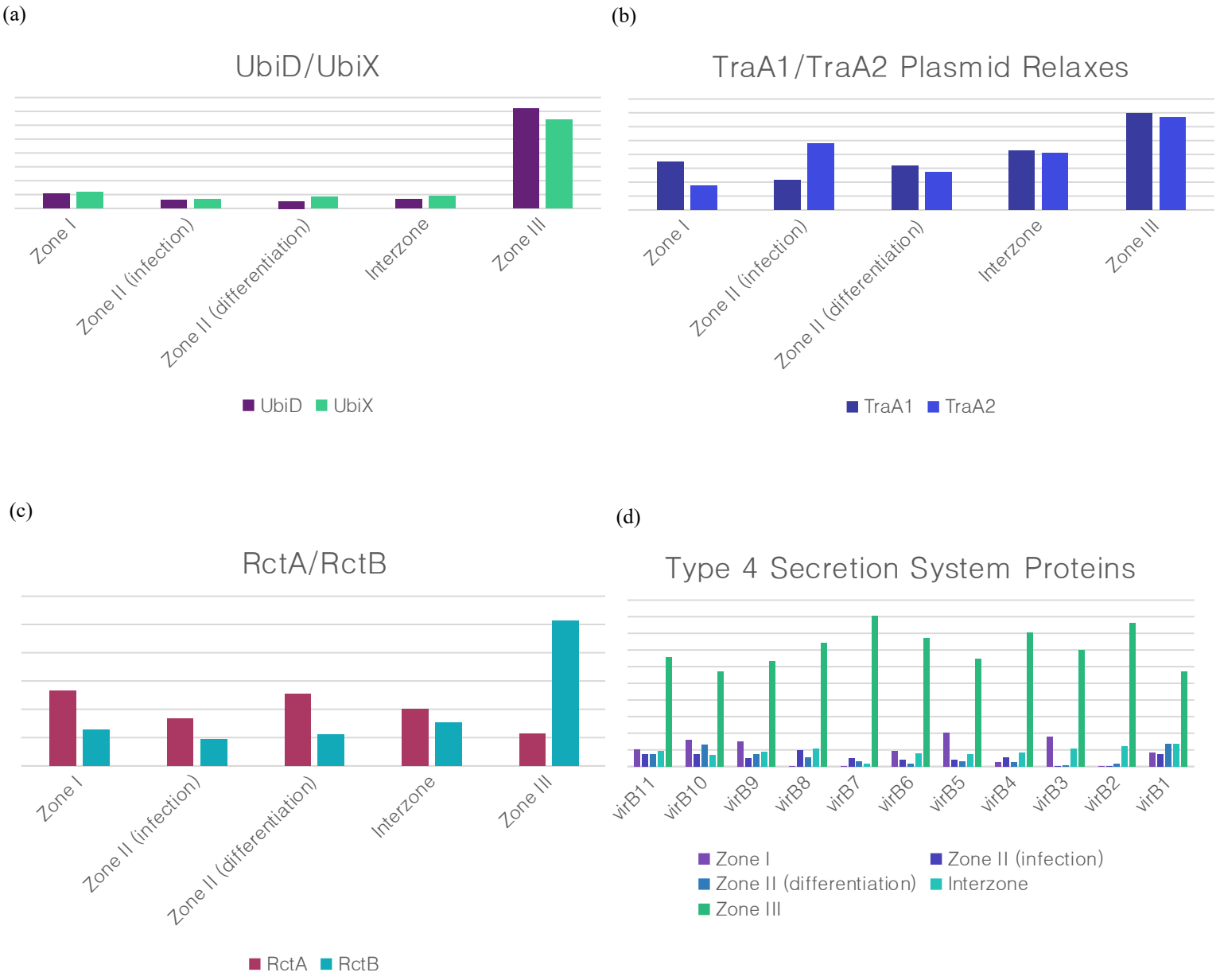


Figure 12. Charts showcasing RNA-seq data of various genes during rhizobia-legume symbiosis within the different zones of root nodules. (a) UbiD decarboxylase and UbiX flavin prenyltransferase, a cofactor involved in gallate decarboxylase biosynthesis. (b) *traA* genes coding for enzymes controlling conjugative plasmid transfer in bacteria. (c) *rctA* and *rctB,* plasmid relaxase regulators. (d) Type IV Secretion System (T4SS) proteins involved in DNA transfer between bacteria.

8. High dissimilarity in proteins from a distinct region of the symbiotic megaplasmid between *Sinorhizobium* spp. strains

A proteomic comparison of the six strains used was performed as a final test to reach a comprehensive evaluation of this comparative study. The result shows a clear difference in protein sequence in one area of the megaplasmid pSymA, as well as interesting similarities between the strains in other regions of the genome.

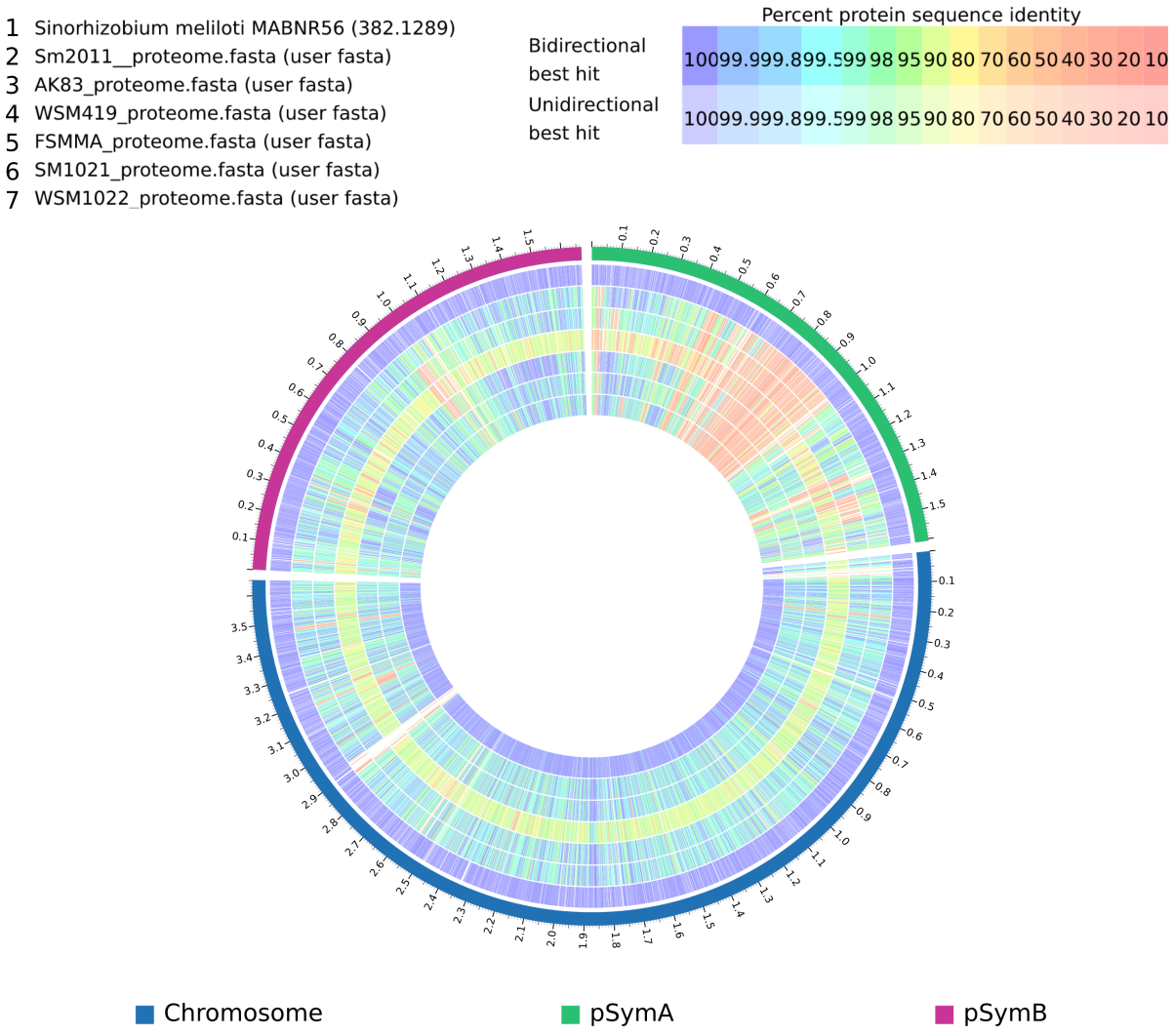


Figure 13. Circular proteome comparison graph. The graph depicts the results of the bidirectional BLASTP analysis of the six strains against a reference genome. Species ordered the outermost to the innermost of the circle: 1) *S. meliloti* MABNR56 2) *S. meliloti* SM11 3) *S. meliloti* AK83 4) *S. medicae* WSM419 5) *S. meliloti* FSM-MA 6) *S. meliloti* 1021 7) *S. meliloti* WSM1022.

Part IV. Discussion

The antimicrobial effect of GA appears to be highly species or even strain-dependent. This suggests the direct involvement of phenolic acids as molecules involved in the selection mechanisms in the symbiosis between rhizobia and legumes. However, given that the strains' susceptibility to GA does not directly correlate with their aptitude for entering and sustaining effective, mutually beneficial symbiotic relationships [121,133], we can surmise that under normal conditions resistance to plant phenolic acids is not the sole determining factor is plant-microbe specificity, but one with a potential role in the intricate molecular interactions between plants and symbiotic bacteria.

Local Alignment Search (BLAST) showed a high degree of conservation of a gene involved in phenolic acid degradation and metabolism across the order *Hyphomicrobiales*. The gene encodes the enzyme gallate decarboxylase, which catalyzes the degradation of GA [124]. Depending on the genus, or some cases the species, was present in either chromosomal or plasmidic DNA. Combined with the bioassay results, the selective effect of GA exerted by plants on bacteria could be by reason of differences in gene transcription, mobility, or other mechanism conferring resistance to phenolic acids in bacteria, rather than a gene presence-absence basis.

Synteny analysis revealed differences between the studied *Sinorhizobium* spp. strains in genes controlling conjugal transfer and replication of plasmids. *rctA,* encodes a repressor protein whose role is inhibiting bacterial conjugation, while *rctB*, its antagonist, modulates the activity of *rctA* and thus promotes plasmid transfer between bacteria [132]. *rctA* was found to be present in all tested strains, while *rctB* was not present in strains *S. meliloti* AK83 and *S. medicae* WSM419. Interestingly, these two strains were inhibited the most by GA. This could suggest that effectiveness of plasmid transfer is a key determinant of bacteria's resistance to plant phenols and thus the selective pressure of plants.

RNA-seq data analysis presented crucial insight into the relevance of the aforementioned genes during the rhizobia-legume symbiosis. *ubiD* and *ubiX*, genes involved in phenolic acid metabolism in bacteria [124], were considerably up-regulated in the nitrogen fixation zone of the nodule (Zone III). This further supports the idea that phenolic acids, such as GA, act as selective molecules in the process of symbiotic nitrogen fixation (SNF) between Rhizobia and leguminous plants. *traA1* and *traA2* relaxases were gradually up-regulated following the progression of

bacteroid specialization in Zone III, suggesting that plasmid conjugation is a factor of SNF in the symbiosis between *S. meliloti* and *M. truncatula.* r*ctA* and *rctB* exhibit increasingly inverted levels of expression leading up to Zone III of the nodule, where *rctB* expression increases substantially relative to other zones, while *rcA* levels decrease.

Regulation of genes encoding Type IV Secretion System (T4SS) proteins showed a similar trend, where expression levels drastically rise in Zone III of the root nodule compared to other zones. T4SS is known to play a role in horizontal gene transfer (HGT) during bacterial conjugation [134]. This suggests that the regulation of plasmid transfer in *S. meliloti* has a functional purpose in the symbiotic relationship and is directly influenced by the nodulation process.

Constructing the phylogenetic tree using 16s rRNA helped elucidate the evolutionary relatedness of the strains, with most being part of one of two main branches; an earlier branch (*S. meliloti* Ref, *S. meliloti* 2011, *S. meliloti* 2011, *S. meliloti* FSM-MA) and a later branch (*S. medicae* WSM419, *S. meliloti* WSM1022, *S. meliloti* AK83, *S. meliloti* SM11).

The Pan-genome analysis showed a considerable variability in the number of strain-specific and accessory genes among the studied strains of *Sinorhizobium* spp., with *S. medicae* WSM419, *S. meliloti* SM11, *S. meliloti* AK83, and *S. meliloti* WSM1022 being the strains having the most of such genes. The disparity in strain-specific gene count suggests numerous mutations or HGT events along the evolutionary history of the strains. It also helps explains the observed differences in phenotype between these strains, especially in the context of rhizobia-legume symbiosis [115–121].

Plotting the localization of strain-specific genes (**Figure 7.)** provided insight into the potential basis for the observed differences. We can observe that in most strains, strain-specific genes are clustered in specific parts of the two symbiotic megaplasmids, pSymA and pSymB, while the chromosome contains relatively few of such genes. This indicates that the genetic differences in the studied strains lies within these plasmids. Functional annotation of the two plasmids revealed some distinctions between pSymA and pSymB, with the latter having more chromosome-like features. Comparing the proteomes of the studied strains revealed more evidence suggesting pSymA is the region where the most difference between the strains can be observed, further supporting previous findings.

Conclusion

In this work we looked at the selective capability of a gallic acid (GA), a plant phenol, on symbiotic bacteria from the genus *Sinorhizobium*. We found that GA only has an inhibitory effect on certain strains, while others are able to proliferate normally in its presence. To understand the mechanism underlying the resistance or lack thereof to GA by the studied bacteria, a thorough comparison of the genomes of studied strains was carried out. We found that GA resistance does not necessarily correlate with effective symbiosis capabilities in most strains, as well as the indication that the mechanisms conferring resistance are tied to gene regulation and mobility via bacterial conjugation. RNA-seq data analysis presented more evidence tying plasmid transfer to symbiosis. Plasmid relaxases and conjugation regulators exhibit elevated expression during the nitrogen fixation phases of nodulation. The two symbiotic megaplasmids of *Sinorhizobium* spp. contain a large number of strain-specific genes, and display functional variability between strains. Phylogeny revealed distinct lineages, with a clear connection between evolutionary closeness and gene features to symbiotic prowess.

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