2 Elucidation of quorum sensing components and their role in regulation of symbiotically important traits in pigeon pea nodule bacteria

2.1 Introduction

Pigeon pea (*Cajanus cajan*) is a perennial legume of the family *Fabacae*. It is cultivated in more than 20 countries on close to 7 million hectares of land (Saxena *et al.*, 2010; Chanda Venkata *et al.*, 2018). It is the sixth most important legume crop globally (Varshney *et al.*, 2012). With close to 25% protein in the dry seeds, and the aerial parts of the plant serving as fodder, it is an excellent source of N-nutrition not only for humans but also for animals. It has been reported to be nodulated by many genera of rhizobia, including *Bradyrhizobium, Rhizobium* and *Ensifer* (earlier known as *Sinorhizobium*) (Fossou *et al.*, 2016). According to Herridge *et al.* (2008), pigeon pea obtains about 65% of its nitrogen from symbiotic association with rhizobia and thus the rhizobia associated with pigeon pea and their symbiotic efficiency are crucial subjects of investigation. In most rhizobia of diverse genera characterized so far, a large number of physiological processes that determine the effectiveness of such symbiotic associations are regulated as a function of their population density (González and Marketon, 2003).

Quorum sensing (QS) is the mechanism of collective regulation of genes in bacteria in response to population density. The most important type of QS in Gram negative bacteria works by synthesis, accumulation and detection of one or more types of autoinducer molecules, known as N-acyl homoserine lactones (AHLs). These bacteria possess one or more pair(s) of luxI-type AHL synthases and luxR-type response regulators (Papenfort and Bassler, 2016) . These elements of a quintessential QS system collectively govern the relevant cellular activities in accordance with the density of the growing population (Whitehead *et al.*, 2001). Rhizobia typically possess multiple such *luxRI* type circuits, for instance *sinRI* or

ngrRI and *traRI* in *Ensifer* spp.; *cinRI, rhiRI, raiRI* and *traRI* in *Rhizobium* spp.. These circuits are often arranged in a functional hierarchy (Sanchez-Contreras *et al.*, 2007) in the sense that AHLs and activity a 'master' circuit governs those of the other downstream to it. In case of the genus *Ensifer,* SinI is responsible for the production of diverse long-chain AHLs. These AHLs are bound by sinR and leads to the regulation of expression of its own as well as other promoters or are bound by other *luxR* family transcriptional regulators such as ExpR to regulate several other promoters (Hoang *et al.*, 2004). On the other hand, the TraRI system in *Ensifer* is homologous to the same circuit of *Agrobacterium tumefaciens* and has only been reported to regulate the conjugal transfer of host plasmid. A complex but well-coordinated operation of SinR/I and ExpR has been demonstrated to regulate numerous cell-processes in *E. meliloti* such as production of exopolysaccharides, biofilm formation, motility, and chemotaxis etc. (Calatrava-Morales *et al.*, 2018). These processes determine the absolute and competitive colonization of host root by the *Ensifer*, ultimately determining the effectiveness of nodulation (Rinaudi-Marron and González, 2015) and thus are very crucial in understanding of the rhizobial symbiotic physiology. However, most detailed studies of rhizobial QS have mostly been with a few laboratory strains such as *Rhizobium leguminosarum* bv viceae 3841, *E. meliloti* strain 1021 (Calatrava-Morales *et al.*, 2018) and *R. etli* CFN42 (Zheng *et al.*, 2015). Despite the economic and agricultural importance of the pigeon pea and its rhizobia, the molecular studies aimed at understanding symbiotic physiology of these rhizobia are scarce and since QS plays a central role in its regulation, this part of the study characterizes QS of pigeon pea rhizobia in detail.

The isolated nodule occupants were screened for AHL production and host growth promotion. Genetic components of QS, chemical diversity of autoinducers produced, and regulation of symbiotically important phenotypes were characterized in the screened strains. Subsequently, the transcriptional control of genes regulating these traits was assessed. This chapter thus leads to generation of a multicomponent anatomical and operational map of the QS phenomenon in *C. cajan* nodulating *Ensifer* strains.

2.2 Materials and Methods

2.2.1 Bacterial strains and culture conditions

Bacterial strains used in this study have been enlisted in Table 2.1. The rhizobial isolates were routinely grown on Tryptone yeast extract (TY) medium (6 g/l Tryptone, 3 g/l yeast extract) with added 3mM $CaCl_2$ at 30°C unless when mentioned otherwise. *Agrobacterium tumefaciens* NTL4 (pZLR4) (Luo *et al.*, 2003) was employed as AHL biosensor and was propagated on Luria Bertani medium supplemented with gentamycin (20 µg/ml) for maintenance and grown in AB minimal medium (Chilton *et al.*, 1974) for assays. *Ensifer meliloti* strain 8530 (Pellock *et al.*, 2002) was used as positive control in AHL bioassays and was propagated in a manner similar to other rhizobial strains with supplementation of streptomycin (100 ug/ml).

Bacterial strain	Relevant characteristics	Reference
Pigeon pea-nodule bacteria isolated in this study		
FP121, FP131, FP141, FP142, FP162, FP163, FP191	Isolated from Farm 1 of Fatepur, Gujarat, India	This study
FP 272, **FP 291**, FP 292	Isolated from Farm 2 of Fatepur, Gujarat, India	
HP 111, HP 112, **HP 113**, HP 114, HP 117, HP 118, HP 119, HP 120, HP 121, HP 123, HP 125, **HP 127**, HP 128	Isolated from a farm of Hansapura, Gujarat, India	
Sh131, Sh178	Isolated from a farm of Shahpura, Gujarat, India	
Su141	Isolated from a farm of Sundarpura, Gujarat, India	
Sa111, Sa112, Sa113	Isolated from a farm of Salat, Gujarat, India	
MF 121	Isolated from Model farm, Pulse Research Inst., Anand	

	Agricultural University, Vadodara, Gujarat, India	
Other Standard laboratory strains		
Agrobacterium tumefaciens NTL4 pZLR4	AHL biosensor strain	Cha *et al.*, 1998; Luo *et al.*, 2003
Ensifer meliloti 8530	Alfalfa nodulating WT strain, ($expR^+$ version of Strain 1021), well studied QS positive strain	Pellock *et al.*, 2002; Gurich and Gonzalez, 2009

Table 2.1 Bacterial strains used in the study. The isolates highlighted boldface as well as underlined are the strains that were selected for detailed characterization after the screening (identified to belong to the genus *Ensifer*).

2.2.2 Isolation of bacteria from pigeon pea nodules

The isolation of nodule bacteria was done as described in (Vincent, 1970). Following are the details of the procedure. In order to isolate rhizobia nodulating pigeon pea, the pigeon pea plants were collected from the agricultural fields around Vadodara, Gujarat, India. Collection was done only from the soils in which no bacterial inoculation was done. Plants were cut just above the crown and were brought to the lab. After washing the root with water, several nodules were plucked off the root carefully ensuring no damage to their integrity. The nodules were subsequently cleaned topically by a wash with 70% ethanol which was preceded by two vigorous and followed by two brief washes with sterile distilled water. Surface sterilisation was done by swirling and gently vortexing the topically cleansed nodules in 0.1% mercuric chloride for 45 seconds, followed by five thorough washes with sterile distilled water, each 30 sec long. Surface sterilized nodules were crushed in 50 µl of sterile Yeast extract mannitol (YEM) broth (in g/L; mannitol 10.0, yeast extract 1.0, $MgSO_4.7H_2O$ 0.2, NaCl 0.1, K_2HPO_4 0.5), a loopful of which was inoculated on a YEM agar plate and incubated for 2 to 7 d. Grown bacterial colonies were subcultured and maintained on TY medium.

2.2.3 Plant experiments

In order to screen the nodule isolates for their proficiency in nodulation and for their effect on the host growth, pot-level plant experiments were set up as described by Vincent (1970) with following specifications. Seeds of pigeon pea (Variety BDN-2, obtained from Pulse Research Station, Anand Agricultural University, Model Farm, Vadodara, India) were surface sterilized as that described for nodules in the previous section. Surface sterilized seeds were allowed to germinate for ~48 h in petri plates containing sterile 0.7% water agar in darkness till the radicle attained about 2.5-3 cm of length. In order to ensure equal amount of bacteria per seedling, germinated seedlings of similar length were used. They were then soaked in cell suspensions of different bacterial strains adjusted to ~10^8 cfu/ml, for 4 h at 30 °C in the dark. Each pot containing 3 kg of sterilized soil was sown with three bacterized seeds coated with the same bacterial isolate. Pot with unbacterized seeds was used as the control. The plants were maintained in green house with 12 h dark-light periods. At the end of 28 days, plants were harvested and various parameters such as nodule numbers, root and shoot weight and lengths were reported.

2.2.4 Detection of Siderophore production

Detection of siderophore production was carried out using Chrome azurol -S (CAS) agar plate as described by Schwyn and Neilands, (1987). For the CAS assay all the glassware were cleaned with 6M HCl *a priori*. Double distilled water was used for making all the reagents. In order to prepare CAS dye, 60.5 mg CAS was dissolved in 50 ml water and subsequently mixed with 10 ml iron (II) solution (1 mM $FeCl_3.6H_2O$, 10 mM HCl). Under stirring conditions, this solution was slowly added to 72.9 mg HDTMA (Hexadecyltrimethylammonium bromide) which was dissolved in 40 ml water. This gave rise to a dark blue liquid was autoclaved. A basal medium consisting of 750 ml H_2O , 100 ml 10X MM9 salts , 15 g agar,30.24 g Pipes , and 12.00 g of a 50% (w/w) NaOH was autoclaved separately. Thirty millilitres of casamino acids (l0%), and other required supplements like vitamins and antibiotics were added as sterile solutions once the main solution was cooled down sufficiently. The CAS dye solution was added to the autoclaved agar along

the glass wall. Each plate was prepared with 30 ml of this blue agar.Rhizobium specific ingredients were as follows and added after the autoclaving: 10 ml glucose (20%) as a carbon source, 5 ml L-glutamic acid (10%, neutralized), and 2.5 ml (+)-biotin (0.02%).The composition of 10X MM9 salts: (In g%) Na_2HPO_4.7H2O 0.15; KH_2PO_4 0.15; NaCl 0.5, NH_4Cl 1.0. Additionally, 1ml each of 1M MgSO4 and 0.1M CaCl2 were also added.

2.2.5 Quantification of Indole-3-acetic acid production

Indole-3-acetic acid (IAA) estimation was done as described in Loper and Schroth (1986) with following details. Cells were grown in the following minimal medium (in g%; Na2HPO4.7H2O 0.15, KH2PO4, 0.15, NaCl, 0.5, NH4Cl 1.0) with 5% glucose as C-source. The medium was autoclaved at 10psi for 20 minutes. To the autoclaved cooled medium 1 ml each of 1M $MgSO_4$.7H2O and 0.1M $CaCl_2$ that were prepared and autoclaved separately were added. A loopful of overnight grown bacterial culture was inoculated in 2ml minimal medium amended with and without 50µg/ml tryptophan. Cultures were shaken for 48h at 30°C. Fully grown cultures after ~30h growth were centrifuged at 12000g for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Samples were incubated for 25 minutes at room temperature. Development of pink colour indicates IAA production which was quantitated by measuring OD at 530 nm with the help of spectrophotometer. Concentration of IAA produced by cultures was determined with the help of standard graph of IAA (Hi-media) obtained in the range of 10–100 µg/ml.

2.2.6 Qualitative detection of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production

The nodule isolates were checked for the presence of ACC deaminase by testing their ability to grow on 1-aminocyclopropane-1-carboxylic acid as the sole N-source. Plates containing DF salts minimal medium (Dworkin and Foster, (1958)comprised of Glucose, 2.0 g; gluconic acid, 2.0 g; citric acid, 2.0 g; KH_2PO_4,

4.0 g; Na_2HPO_4, 6.0 g; $MgSO_4.7H_2O$, 0.2 g; micronutrient solution $CaCl_2$, 200 mg; $FeSO_4$.7H2O, 200 mg; H_3BO_3, 15 mg; $ZnSO_4$.7H2O, 20 mg; Na_2MoO_4, 10 mg; KI, 10 mg; NaBr, 10 mg; $MnCl_2$, 10 mg; C_OCl_2, 5 mg; $CuCl_2$, 5 mg; $AlCl_3$, 2 mg; $NiSO_4$, 2 mg; distilled water, 1000 ml) with no N-source was used as negative control and those with $(NH_4)_2SO_4$ as positive control. ACC as the sole N-source was used at 3mM final concentration in the plate. The plates were incubated at 30ºC for 72 h. Growth on ACC supplemented plates was compared with negative and positive controls and was based on growth.

2.2.7 Bioassay for N-acyl homoserine lactone production

Bacterial isolates were screened for production of AHL molecules by employing one of the most versatile AHL-biosensor, *Agrobacterium tumefaciens* NTL4 (pZLR4) using petri plate based assay as described by Ravn *et al.* (2001). This sensor strain is capable of detecting a broad range of AHLs at high sensitivity (Steindler and Venturi, 2007) and has also been employed for detecting rhizobial long chain AHLs (Marketon and González, 2002). The detection strategy followed for screening bacterial producing AHLs is as follows. The spent culture supernatants of the nodule isolates grown in TY broth until late log phase were spotted onto a plate containing X-gal and the biosensor, as well as onto a plate lacking the biosensor. *Ensifer meliloti* 8530 and *A. tumefaciens* NTL4 mutant incapable of producing AHLs were used as positive and negative control respectively. A blue zone around the spot in the plate containing biosensor and its absence in the plate devoid of the biosensor following an overnight incubation were considered a true positive result.

2.2.8 Taxonomic identification of bacterial strains

Bacterial isolates were identified taxonomically using PCR amplification and sequencing analysis of 16S rRNA and recA gene fragments. PCR amplification of the genes was carried out from the isolated genomic DNA of isolates. The genomic DNA was isolated using CTAB method of genomic DNA miniprep (Wilson, 2001). Amplification of 16S rDNA was done using the universal eubacterial 16S primers

27F and 1541R (Pillai and Archana, 2008) and that of *recA* was performed using primers reported by Gaunt *et al.* (2001). Amplified products were gel purified and were sequenced at Agrigenome labs Pvt Ltd., Bengaluru, India. Obtained sequences were used for alignments on NCBI BLAST(Altschul *et al.*, 1990), Ribosomal Database Project (RDP)-Classifier tool (Wang *et al.*, 2007), EzTaxon (Chun *et al.*, 2007) as well as for generating phylogenetic tree using MEGA X (Kumar *et al.*, 2018).

2.2.9 Extraction of AHLs from spent bacterial culture supernatants

AHLs produced by the bacterial isolates were extracted using liquid-liquid extraction of their late-log phase culture-supernatant with ethyl acetate as described in Shaw *et al.* (1997) with following details. Isolates were grown in 500 ml of Tryptone yeast extract broth in a 1.5 L Erlenmeyer glass flask up to OD 1.0 in shaking conditions. At the end of growth, the culture supernatants were separated by centrifugation of the culture at 12000 rpm for 10 min and extracted in 1:1 ratio with HPLC-grade ethyl acetate pre-acidified with glacial acetic (0.2% v/v) acid. This was repeated one more time with the supernatant and the ethyl acetate fractions were pooled. Ethyl acetate was subsequently evaporated using rotary evaporator (Rotavapor R-100, BUCHI, Switzerland) and the AHL extract was finally dissolved in methanol. AHL extracts prepared this way were stored at -20°C with only infrequent withdrawals. For gene expression analysis, a dummy extract was prepared the same way as above but from 500 ml uninoculated media, and was used as control against the sets that received AHL treatment.

2.2.10 Chemical Identification of AHLs using Liquid chromatography tandem mass spectrometry

AHL extracts prepared and dried as described above were dissolved in 2 ml of HPLC-grade methanol prior to the injection in ACCUCORE® C18 150 X 2.1, 3 µm RP column on Acquity® UPLC (Waters, USA). The parameters for liquid chromatography (LC) were employed as follows: total run time was 20 min at a flow rate of 250 µl/min, and a linear gradient of [100% Acetonitrile]:[0.1 % formic

acid + 95:5 H2O:Acenonitrile] was applied for elution. The eluents were subjected to mass analysis in Acquity® TQD (Triple Quadrupole Detector) mass spectrometer (Waters, USA). The analysis was performed in Multiple Reaction Monitoring (MRM) mode which is widely applied in AHL profiling, due to its high sensitivity and the provision to yield quantitative data (Gould *et al.*, 2006; Ortori *et al.*, 2014). The MS/MS parameters were set as follows: ion spray voltage of 3.5 kV, declustering potential of 30 V, source temperature of 120°C and desolvation temperature of 350°C. Ionization was achieved by electron spray ionizer and the polarity of monitoring was positive while N2 was used as the collision gas. Six AHL standards (Sigma Aldrich, USA and Caymen chemicals, USA) were taken for validation.

2.2.11 Detection of quorum sensing gene homologues in *Ensifer* isolates

Degenerate primers for the detection of homologues of *sinI, sinR, expR* as well as *traI* and *traR* from nodule isolates were designed by targeting against the conserved regions based on the alignments of sequences obtained from related rhizobial strains. Table 2.2 lists all the primer pairs, their annealing temperatures and expected amplicon sizes. PCR mixture contained, in addition to the buffer, 1.5 mM $MgCl_2$, 0.5mM each of dNTP, 0.4 µM of each primer and 1-5 ng DNA template. Cycling conditions for PCR were as follows: Initial denaturation: 95°C-5'; 35 cycles of 95°C-30s, T_a-45s, 72°C-1'; 72°C-5' (where T_a indicates annealing temperature which is specified for each primer pair in Table 2.2). Amplicons obtained were sequenced using the services of Agrigenome labs Pvt Ltd., Bengaluru, India, and homology analysis was carried out using NCBI BLAST. The resultant sequences were submitted to NCBI GenBank for public availability. Table S1 lists the accession numbers of these NCBI records.

Primer	Sequence (5'→3')	Annealing temp. (°C)	Amplicon size# (bp)
Used for detection of QS circuits			
sinI F sinI-R	CAGGCCATCGACGARATGTTCCGGCTGCG TCGAGMACGGTGCCCTCGATGG	65	566
sinR-F sinR-R	GRCTAATCAACAGGCTGTCCT GCGCWACMGCTTGCGTGTTGGA	55	701
traI-F traI-R	TCGSCVAKSCGCTGAAGCGGCCA MRHCATCACSARCTRCGCGC	55	443
traR-F traR-R	CGRTCCTTGGCRATTGCMACAAG GCTCGCTCRTCGAYATGYTGGAAGC	66	676
expR-F expR-R	TGGAAGAGATGAAGACGCGCGAGG TTGCCCATGAGCCCGCGCCG	71	370
Used for qRT-PCR			
aglE-F aglE-R	GCCGACCTGAAGTTCAAGCC CGCTCTTGAACAGAGCCTCG	60	145
cheY1-F cheY1-R	CTGACTGTGGACGATTCCCG GCATGTTGATGTCGGTGACG	60	154
flgD-F flgD-R	ACCCTGAACTATCAGAGCTTCC CGAGAAGGTCGCAAGCTGC	60	111
ndvA-F ndvA-R	AGCGCTTCAGTATCTTGCTGTC GAACGAAGGCGATCGTATTGAAGAC	60	196
expE6-F expE6-R	CCATGCGGGTGGGTTTCTCG CTGGGCGTCGACAATATCTTTCA	60	224
pilA1-F pilA1-R	TCGCCCGCCTGATGAAGG CCGTCGCGCCGGTGATCAG	60	86
sinI-qF sinI-qR	GGGCCGAATATGCTGGACGA CACACATGAGCTCCGCAGC	60	163
sinR-qF sinR-qR	ACCTGTCGAATGGACGACCG CGACATGTTGGCGTTGATGGC	60	157
16S -F 16S-R	TCCTACGGGAGGCAGCAGT CCGTCATTATCTTCACCGGTG	60	120

Table 2.2 Primer pairs used in this study for PCR-detection of gene circuits and qRT-PCR The degenerate bases are designated using the standard IUPAC nomenclature as follows: R=A/G, Y=C/T, M=A/C, K=G/T, S=G/C, W=A/T, H=A/T/C, B=G/T/C, D=G/A/T, N=A/C/G, V=G/A/C; #Amplicon size refers to the expected length of PCR product based on gene sequence alignment

2.2.12 Preparation of cells of differential densities and AHL-induction for gene expression analysis

Bacterial culture was serially passaged down to low cell density for expression analysis as suggested by Gao *et al.* (2005) with following details. Respective rhizobial was first grown to OD_{600} of 0.5 in 5 ml TY broth in a tube. From this culture, harvested and washed cells were used to inoculate fresh TY broth which was in turn grown to 0.2 OD_{600}. Similar passage was done subsequently to obtain a final culture suspension of 0.02 OD_{600}. These cells (from 500 ml culture) were washed with and resuspended in 1 ml N-saline (0.85% NaCl w/v). This sparse cell-suspension is referred to as "Low Density Cells" (LDC) hereafter. Another culture was grown to an OD_{600} of 1.0 and the cells were washed with and resuspended in N-saline to yield approximately 10^8 cells /ml. This dense cell-suspension is referred to as "High Density cells" (HDC) hereafter. HDC was used as one of the control sets for AHL induction experiments. For AHL induction, the LDC were incubated for 3 h according to Chen *et al.* (2003) with gentle shaking at 30°C with either its own AHL-extract— prepared as described in Section 2.2.9, or with 0.5 µM of synthetic C16:1-HSL (Caymen chemicals, USA). This gave rise to four sets: (i) LDC- that was treated with dummy extract (Prepared as given in Section 2.2.9) and is supposed to be in "Quorum-off"- state; (ii) HDC- which represents the "Quorum-on" state of high density culture; (iii) LDC treated with AHL extract of the strain itself- to test its effect on the gene expression and phenotypic outcomes; and (iv) LDC treated with 0.5 µM C16:1-HSL- as a standard positive control treatment.

2.2.13 Biofilm formation assay

Strains were tested for their biofilm formation capability on the polyvinyl chloride microtitre plate as suggested by Fujishige *et al.* (2006) with following modifications. The cells were serially passaged to low density as described in Section 2.2.12. Each well in the plate contained 200 µl of LDCs. While untreated controls received dummy extract, in the test sets, the LDCs were treated with AHL extract of the respective strain or 0.5 µM C16:1-HSL in the presence or absence of

250 µg/ml vanillin- a QS based inhibitor of biofilm formation (Choo *et al.*, 2006, Ponnusamy *et al.*, 2009). The plate was incubated at 30°C in static conditions only for 24 h [as opposed to 48-72 as described in Fujishige *et al.*, (2006)] to prevent the AHLs influx in late log phase. Prior to staining for biofilm, OD_{600} of the planktonic biomass was measured. The biomass was then drained and the wells were washed to remove cells. The wells were subsequently air-dried and the biofilms were stained with 0.01% crystal violet for 20min. Spectrophotometric reading of the microtiter plate was done using Tecan InfinitePro 2000 microtiter-plate reader. Each set was performed in triplicates and this was repeated thrice as individual experiments.

2.2.14 Chemotactic motility assay

Modified capillary assay was performed to assess the role of QS in regulation of flagellar motility in response to chemical stimulus in the *Ensifer* strains. The assay method was adapted from Mazumder *et al.* (1999). 2 ml microcentrifuge tubes were used as resrvoirs containing 200 µl of Low Density Cells in chemotaxis buffer (6.1 mM K2HPO4 + 3.9 mM KH2PO4) in the presence or absence of the induction by either respective AHL extract of the strain or 0.5 µM C16:1-HSL. Control set of LDC were treated with dummy extract (Section 2.2.9). 1 mM Proline, as originally suggested by Gotz *et al.* (1982) dissolved in chemotaxis buffer was used as the chemoattractant in the syringe with its end in the cell reservoir. No proline was added in chemotaxis buffer of the syringe in case of negative control for all the sets. The immigrant cells from the capillary were recovered and CFU were counted via dilution-plating on TY agar plates and the values of revcovered CFU were compared across sets.

2.2.15 Real-time quantitative reverse transcription PCR (qRT-PCR)

From each of the four sets prepared as in Section 2.2.12 [(i) LDC- treated with dummy extract, (ii) HDC, (iii) LDC treated with AHL extract of the strain itself, and (iv) LDC treated with 0.5 µM C16:1-HSL], ~10^8 cells were taken and lysed by treating with with 0.5 mg/ml lysozyme for exactly 5 minutes at 30°C. The cell lysis was done immediately following the induction and prior to RNA isolation which

was performed using TRIzol reagent (RNAiso Plus, Takara Bio, Inc, Japan) as per the reagent manual. Briefly, to 1ml cell lysate 1ml of TRIzol reagent was added and mixed well. This was then centrifuged at 12000g for 15 min at 4ºC. The supernatant was then transferred to a fresh vial and 200µl of chloroform was added to it followed by an incubation in ice for 30 min with intermittent gentle mixes. Following the incubation, the mixture was spun at 12000g for 15 min at 4ºC and the aqueous phase was transferred to a fresh 1.5 ml vial. 500µl of isopropyl alcohol was then added to the aqueous phase followed by an incubation at 4ºC for 45 min. The solution was centrifuged at 12000g for 15 min at 4ºC and the obtained pellet was washed with 70% ethanol twice. The pellet was allowed to air dry on the bench. The pellet was then dissolved in DEPC treated sterile Milli-Q water. This was followed by DNase treatment with "Ambion- DNA-free" kit according to its manual. RNA was checked for the quality and integrity by agarose gel electrophoresis and was quantified spectrophotometrically. RNA (1µg) from each set was subjected to cDNA synthesis using PrimeScript™ cDNA Synthesis Kit (Takara Bio, Inc, Japan). qRT-PCR was performed on Roche Lightcycler96 with Takara SYBR mix kit (Takara Bio, Inc, Japan) for the detection chemistry. Oligonucleotide primers used for qRT-PCR are listed in Table 2.2. The cycling conditions were as follows: 95°C-100s; 45 cycles of [95°C-10s, 60°C-30s, 72°C-30s]. Relative quantification of Cq values was done for all genes for each of the sets. Transcript abundance was first normalised using 16S rRNA gene as the endogenous house-keeping control (ΔCq). This was followed by their comparison across treatment sets (ΔΔCq). The relative difference in the transcript abundance is expressed as fold change ($2^{-\Delta\Delta Cq}$) as suggested by Livak and Schmittgen (2001).

2.2.16 Data analysis

Plant experiments data was represented as mean ± S.D. whereas in the phenotypic and transcriptional regulation experiments, the data is shown as mean ± S.E.M. Data was subjected to either Student's unpaired t-test or One Way ANOVA followed by Bonferroni post-hoc test using GraphPad Prism 6.01 (Graphpad Software, California, USA) for significance analysis. Comparisons with p-value less than or equaling 0.05 were only considered as statistically significant.

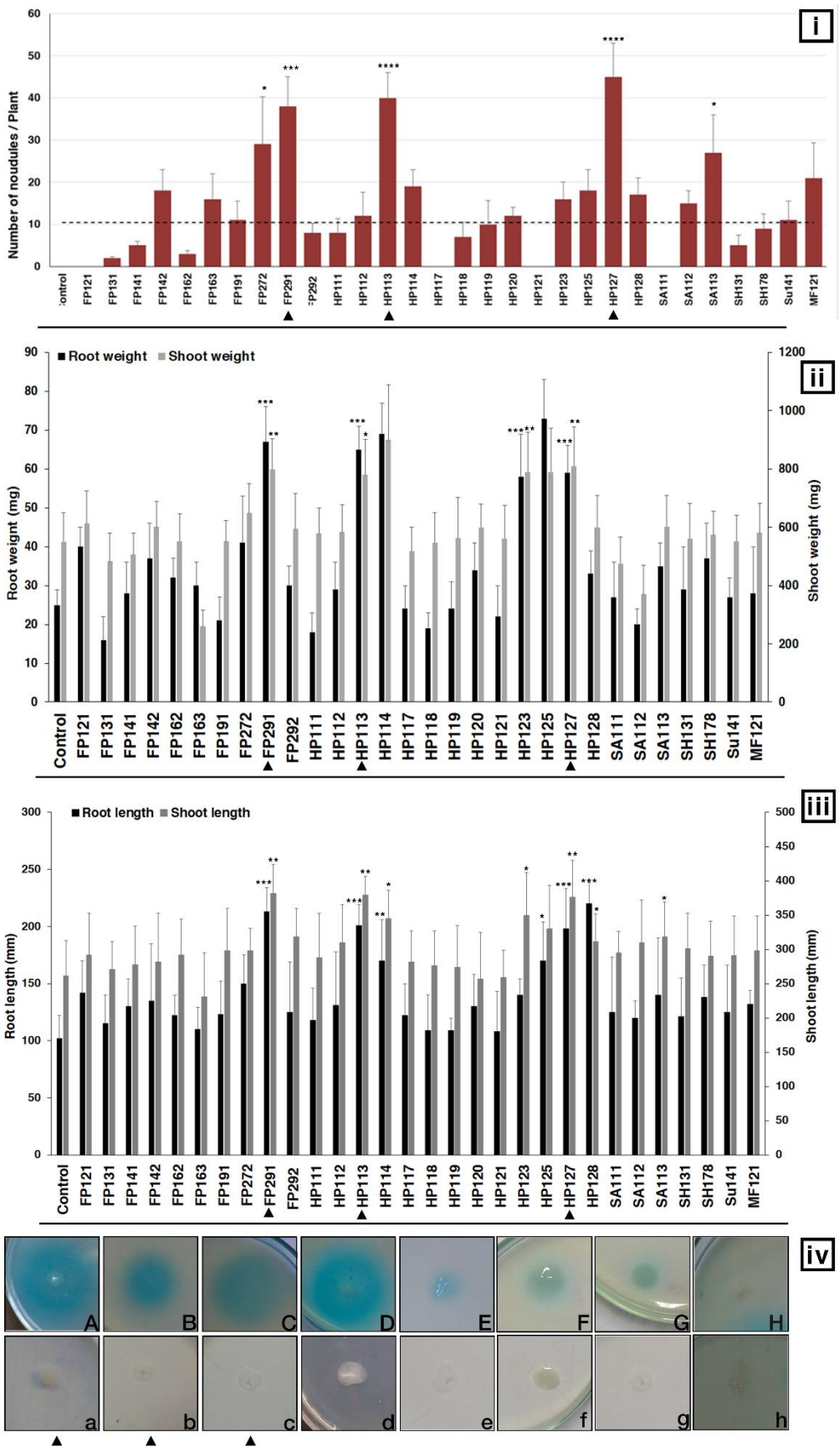
2.3 RESULTS

2.3.1 Isolation of nodule bacteria from pigeon pea

Thirty nodule bacteria were isolated from plants of pigeon pea grown in different fields in and around Vadodara, Gujarat, India. A list of all the isolates and their site of isolation is given in Table 2.1. These isolates were confirmed to be rhizobia by nodulation test on pigeon pea in pot based experiments in gnotobiotic conditions. As shown in Figure 2.1, all the isolates but four developed nodules on pigeon pea plants growing in pots, whereas the control where no inoculation was done, showed no nodules.

2.3.2 Screening for host growth promotion and AHL production

Among the nodulating isolates, five strains – FP272, FP291, SA113, HP113 and HP127 showed relatively greater nodulation efficiency (>25 nodules/plant). The isolates FP291, HP113, HP114, HP123, HP125 and HP127 showed significant plant growth promotion as indicated by a higher weight and length parameters of the plants (Figure 2.1). On screening for QS occurrence, as shown in Figure 2.1 the strains FP291, FP292, HP113, HP125, HP127 and Sh178 showed distinct blue colored zone in the test plates and no color in the control plate indicating that they are quorum sensing positive isolates. Considering the efficient nodulation, plant growth promotion as well as proficiency at QS, the three nodule isolates FP291, HP113 and HP127 (marked with arrow heads in Figure 2.1) were selected for further work. These three isolates were also characterized for their other plant growth promotion activities. The three isolates selected from the screening based on the nodulation, promotion of host growth and presence of quorum sensing were characterized for the presence of other PGP activities that can contribute to the augmentation of host health further — they were tested for ability to produce Indole-3-acetic acid (IAA), siderophores and ACC deaminse enzyme. While all the three isolates were found to produce significant amount of IAA, only HP113 and HP127 were found to produce siderophores (Figure 2.1) while none of the isolates tested positive for ACC deaminase production when grown ACC as the sole N-sources.


i
Number of noudules / Plant
Control
FP121
FP131
FP141
FP142
FP162
FP163
FP191
FP272
FP291
FP292
HP111
HP112
HP113
HP114
HP117
HP118
HP119
HP120
HP121
HP123
HP125
HP127
HP128
SA111
SA112
SA113
SH131
SH178
Su141
MF121
ii
Root weight
Shoot weight
Root weight (mg)
Shoot weight (mg)
iii
Root length
Shoot length
Root length (mm)
Shoot length (mm)
iv
A
B
C
D
E
F
G
H
a
b
c
d
e
f
g
h

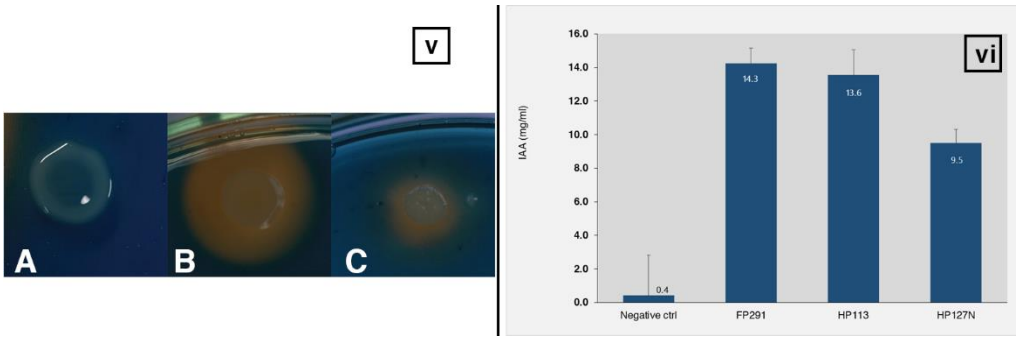


Figure 2.1 Screening of pigeon pea nodule isolates. (i) Nodulation on pigeon pea plants in pot level experiments; (ii and iii) Plant growth promotion (root and shoot weight/lengths); (iv) Production of N-acyl homoserine lactone signals via biosensor mediated plate-based qualitative detection. (v)FP291(A), HP113(B) and HP127(C) on CAS plates for detection of siderophores production. (vi)Estimation of production of indole-3-acetic acid by FP291, HP113, HP127. The dashed horizontal line in Panel-i represents mean of nodule numbers for all the strains. In Panel- ii and iii, the vertical bars represent means of at least three replicates with standard deviation as error bars. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Panel-iv: Upper row shows test AHL indicator plates for bacterial strains FP291 (A), HP113 (B), HP127 (C), HP125 (D), FP292 (E), Sh178 (F), *E. meliloti* 8530 (G) and AHL-synthesis-mutant of *A. tumefaciens* NTL4 (H). Bottom row (a–h) depicts the same strains on control plates that do not contain the indicator organism.

2.3.3 Molecular phylogenetic identification of pigeon pea nodule isolates

In order to discern the identity of the nodule isolates, the 16S rRNA gene fragments of the strains FP291, HP113 and HP127 were amplified with PCR and sequenced. The sequences, when analyzed by RDP-classifier tool, identified all the three strains to be belonging to the genus *Ensifer* (earlier known as *Sinorhizobium*) with 100% confidence. With EzTaxon, all the three isolates showed over 99% similarity with *Ensifer terangae* as well as *E. mexicanus* and *E. chiapanecum*. A neighbor-joining tree of the 16S rRNA gene sequences (Figure 2.2) shows that the isolates cluster with *E. terangae*. In order to gain further insight into the identity of the isolates, recombinaseA gene fragments of the three strains were sequenced. The phylogenetic tree generated from *recA* sequences (Figure2.2) shows that the bacterial isolates are clades of *E. terangae*, *E. mexicanus* and *E. chiapanecum* branch.

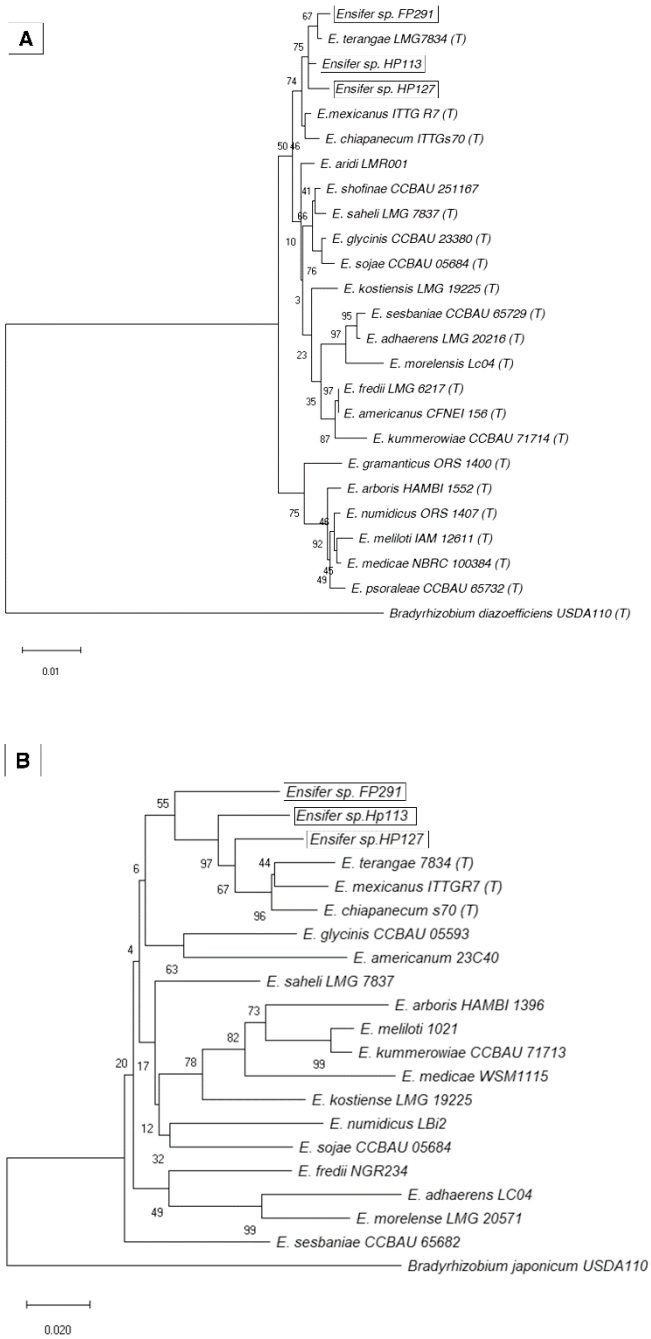


Figure 2.2 Neighbour Joining trees of 16S rRNA gene sequences (top) and recA sequences (bottom) of *Ensifer* strains isolated for this work and of the related strains. Bootstrap values at nodes indicate an average of 1000 replicates. 'T' in parenthesis indicates type strain. The tree is drawn to scale with evolutionary distances calculated using Kimura 2-parameter method. Isolates of this study are indicated by a box around their names.

Thus, the strains FP291, HP113 and HP127 are denoted as *Ensifer* spp. and are taxonomically closest to *E. terangae.* Genomic sequences of 16S rDNA and recA fragments of the three isolates were submitted to NCBI GenBank, and the Table S1 gives the GenBank accession numbers of the respective sequences. In addition to the three selected isoaltes, 11 other isolates were also identified by sequencing their 16S rDNA gene fragments. Although only the three *Ensifer* spp. (FP291, HP113 and HP127) proficient in PGPR and quorum sensing as described in the previous section were taken forward in the study, these 11 strains were identified to get an insight into the diversity obtained in the nodule isolates. This analysis resulted in the identification of some strains belonging to the non-rhizobial genera such as *Ochrobactrum*, Klebsiella, *Providencia, and Brevundimonas* or non-nodulating rhizobia such as *Rhizobium rosettiformans* and *Rhizobium pusense.* Table S2 shows the similarity parameters of these strain with the closest identified member from NCBI GenBank with BLAST analysis. Their 16S sequences have been included as supplementary TableS3.

2.3.4 Chemical identification of n-acyl homoserine lactones secreted by *Ensifer* isolates

Chemical identities of the n Acyl homoserine lactone (AHLs) autoinducers produced by the *Ensifer* isolates were discerned by subjecting their culture supernatants to liquid chromatography tandem mass spectrometry (LC-MS/MS) in Multiple Reaction Monitoring (MRM) method. In order to ascertain the identity of AHLs with confidence, the following four confirmatory conditions were employed, only complying to *all* of which, was an AHL molecule reported to be present: (i) the *m/z* of detected parent fragment must match with the theoretical $[M+H]^+$of the respective AHL; (ii) on the second ionization, the parent ion must fragment into a lactone ring of *m*/*z* 102, which is characteristic to all the AHLs, and another fragment ion of $[M+H-101]^+$ (where M is the parental mass); if any of the two daughter ions were missing, that AHLs was not considered as detected. (iii) both the precursor peaks must occur at the same retention time; (iv) since all the samples were used with identical running conditions retention time for the same AHLs must

be comparable across the samples and with the standards. With this analysis, multiple AHLs in each of the isolates were identified. Table 2.3 gives the details of the detected AHLs along with the respective fragments, their masses and the peak areas corresponding to the abundance of the respective AHL. The mass spectra of all the AHLs from all isolates are given Figure 2.3. Each AHL can be characteristically discerned from the figure with both its daughter ion peaks shown (indicated as “m/z of parent ion” > ” “m/z of daughter ion”). Retention time and fragmentation patterns of the bacterially secreted AHLs matched with the appropriate standard. *Ensifer* sp. HP127 was found to to produce the highest number of (eight) AHLs including six long chain AHLs while *Ensifer* sp. FP291 and HP113 produced four AHLs each. In addition to the length, the AHLs also varied in the substitutions and degree of unsaturation. Since the MRM also permits relative quantification based on the peak areas, the relative abundance (as % of the maximally produced AHL) calculated on the basis of peak area is presented in Figure 2.4. In all the isolates 3-oxo-C16:1-HSL was the highest produced AHL.

	Detected AHL	m/z of the Q1 parent	m/z of Q3 product ion $[M+ H-101]^+$	m/z of Q3 product ion (lactone ring)	Peak area for Q3 product ion (lactone ring) for each parent ion
Ensifer sp. HP127	N-3-oxo-octanoyl-HSL (3-O-C8-HSL)	242.3	141.2	102.1	821.932
	N-3-oxo-dodecanoyl-HSL (3-O-C12-HSL)	298.3	197.2	102.1	1415.023
	N-3-oxo-tetradecanoyl-HSL (3-O-C14-HSL)	326.3	225.2	102.1	48186.49
	N-hexadecanoyl-HSL (C16-HSL)	340.3	239.2	102.1	14616.717
	N-3-oxo-hexadecanoyl-HSL (3-O-C16-HSL)	354.3	253.2	102.1	94853.742
	N-hexadec-11(Z)-enoyl-HSL (C16:1-HSL)	338.3	237.2	102.1	18502.518
	N-3-oxo-hexadec-11(Z)-enoyl-HSL (3-O-C16:1-HSL)	352.3	251.3	102.1	611661.5
	N-3-hydroxy-hexadecanoyl-HSL (3-OH-C16-HSL)	356.3	255.2	102.1	2577.153
Ensifer sp. FP291	N-3-oxo-dodecanoyl-HSL (3-O-C12-HSL)	298.3	197.2	102.1	459.184
	N-3-oxo-tetradecanoyl-HSL (3-O-C14-HSL)	326.3	225.2	102.1	3632.853
	N-hexadec-11(Z)-enoyl-HSL (C16:1-HSL)	338.3	237.2	102.1	9953.142
	N-3-oxo-hexadec-11(Z)-enoyl-HSL (3-O-C16:1-HSL)	352.3	251.3	102.1	66394.461
Ensifer sp. HP113	N-3-oxo-dodecanoyl-HSL (3-O-C12-HSL)	298.3	197.2	102.1	951.763
	N-3-oxo-tetradecanoyl-HSL (3-O-C14-HSL)	326.3	225.2	102.1	15254.976
	N-hexadec-11(Z)-enoyl-HSL (C16:1-HSL)	338.3	237.2	102.1	46840.094
	N-3-oxo-hexadec-11(Z)-enoyl-HSL (3-O-C16:1-HSL)	352.3	251.3	102.1	375830.84

Table 2.3 N-acyl homoserine lactones detected from pigeon pea nodulating *Ensifer* spp. and their MS/MS transitions

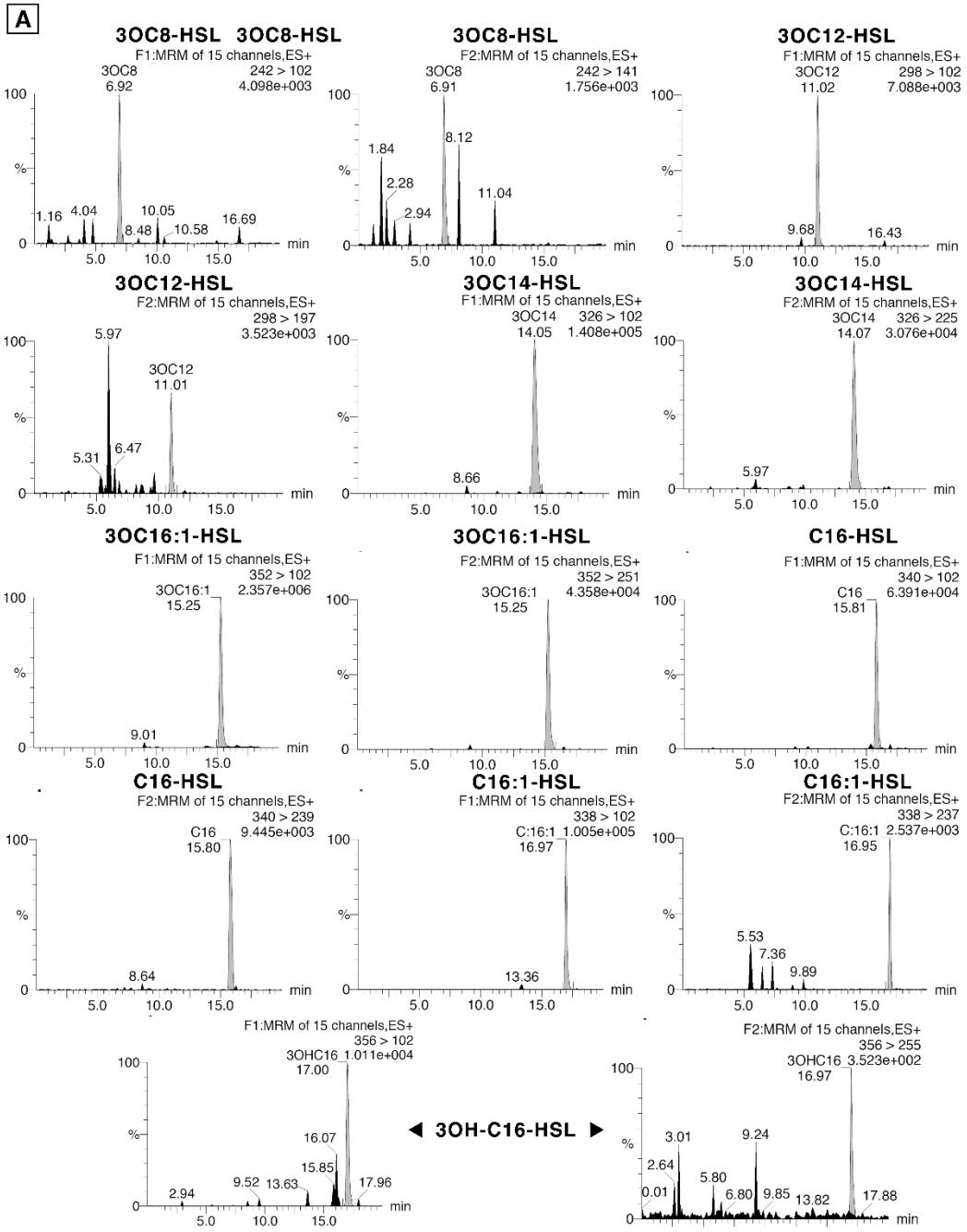


Figure 2.3A LC/MS-MS transitions of n-Acyl homoserine lactones (AHL) detected from *Ensifer* sp. HP127. Each AHL is characterized by two transitions, of which one corresponds to the lactone ring (m/z 102) while the other is unique for each AHL. X-axis indicates the Retention time of the transitioning species while Y-axis indicates intensity.

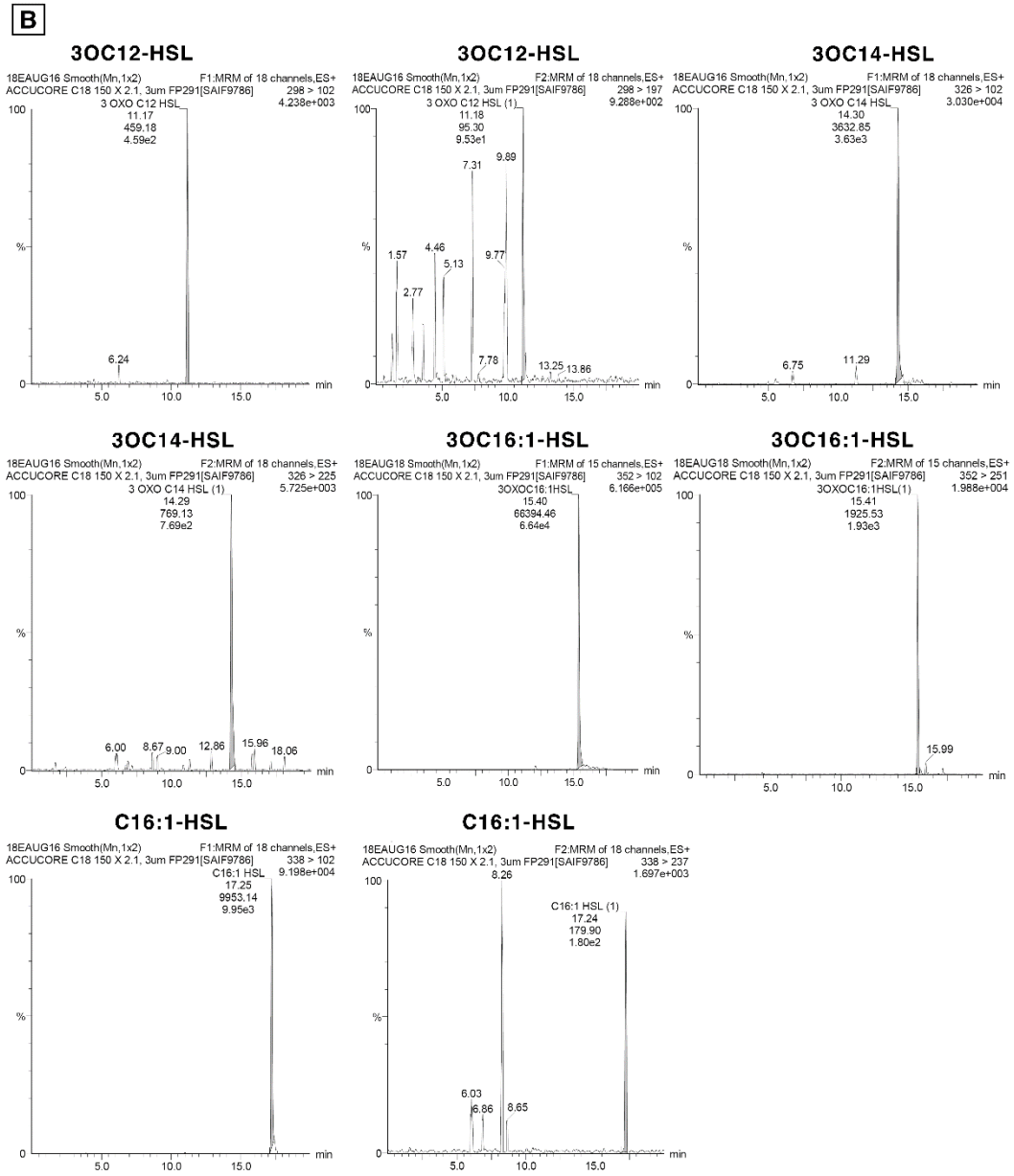


Figure 2.3B LC/MS-MS transitions of n-Acyl homoserine lactones (AHL) detected from *Ensifer* sp. FP291. Each AHL is characterized by two transitions, of which one corresponds to the lactone ring (m/z 102) while the other is unique for each AHL. X-axis indicates the Retention time of the transitioning species while Y-axis indicates intensity.

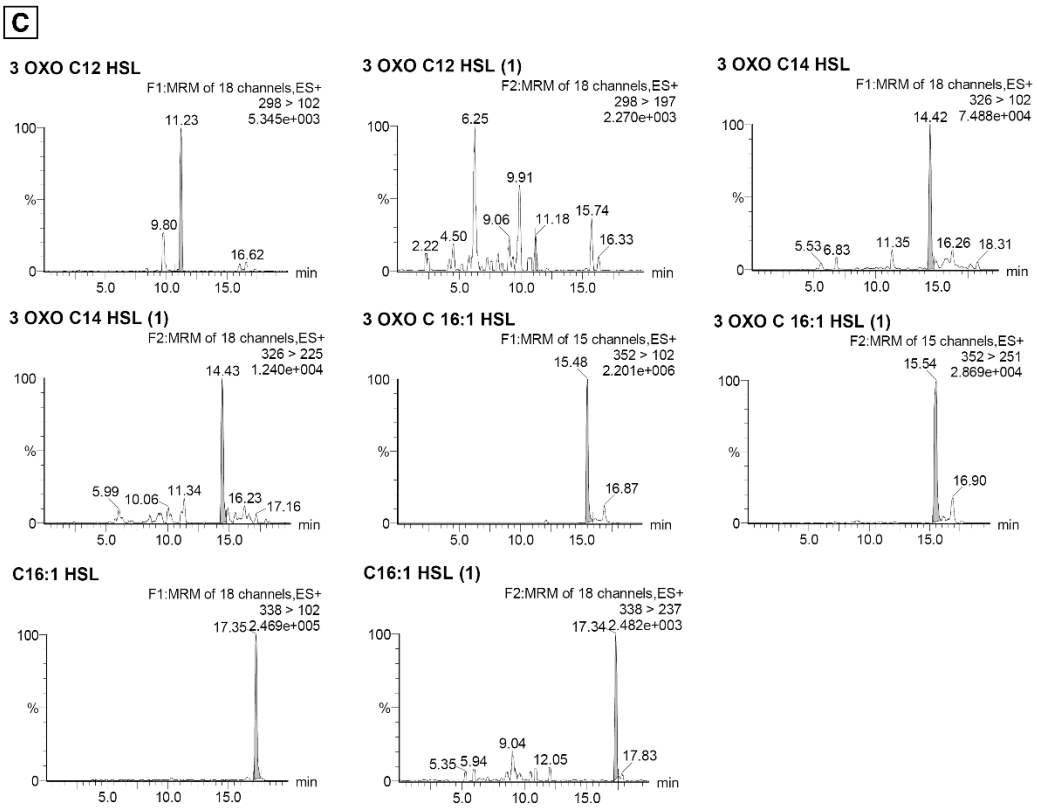


Figure 2.3C LC/MS-MS transitions of n-Acyl homoserine lactones (AHL) detected from *Ensifer* sp. HP113. Each AHL is characterized by two transitions, of which one corresponds to the lactone ring (m/z 102) while the other is unique for each AHL. X-axis indicates the Retention time of the transitioning species while Y-axis indicates intensity.

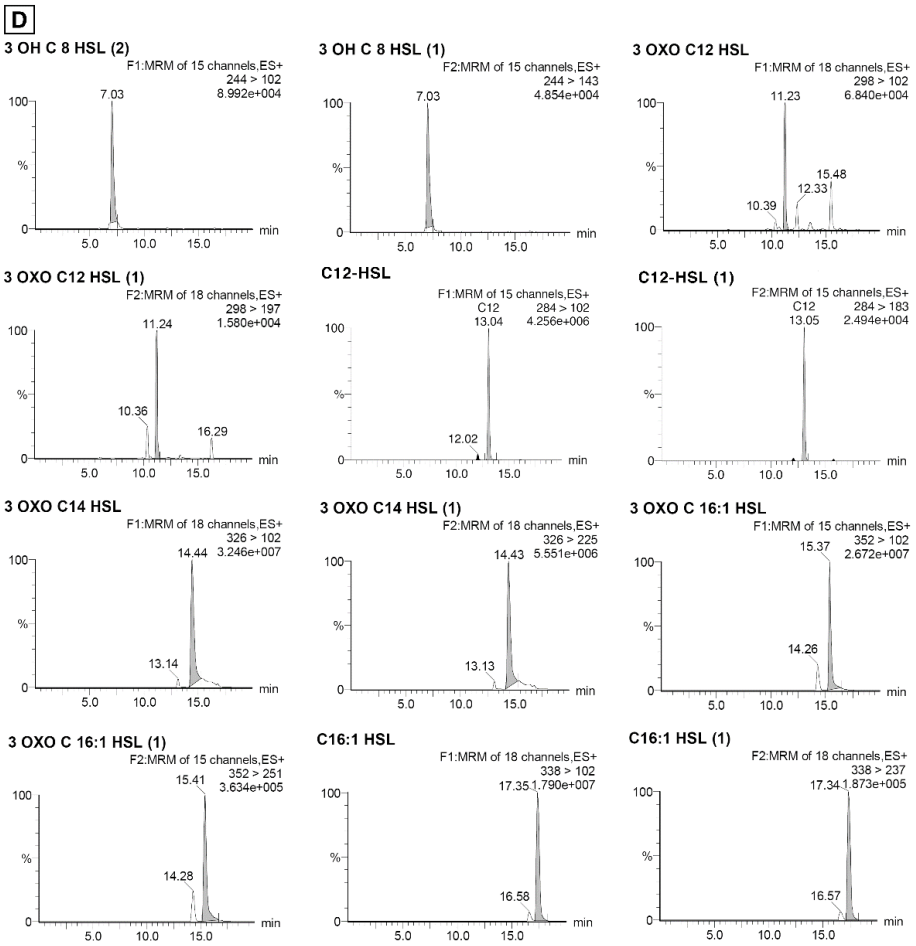


Figure 2.3D LC/MS-MS transitions of n-Acyl homoserine lactones (AHL) detected from the mixture of synthetic AHLs. Each AHL is characterized by two transitions, of which one corresponds to the lactone ring (m/z 102) while the other is unique for each AHL. X-axis indicates the Retention time of the transitioning species while Y-axis indicates intensity.

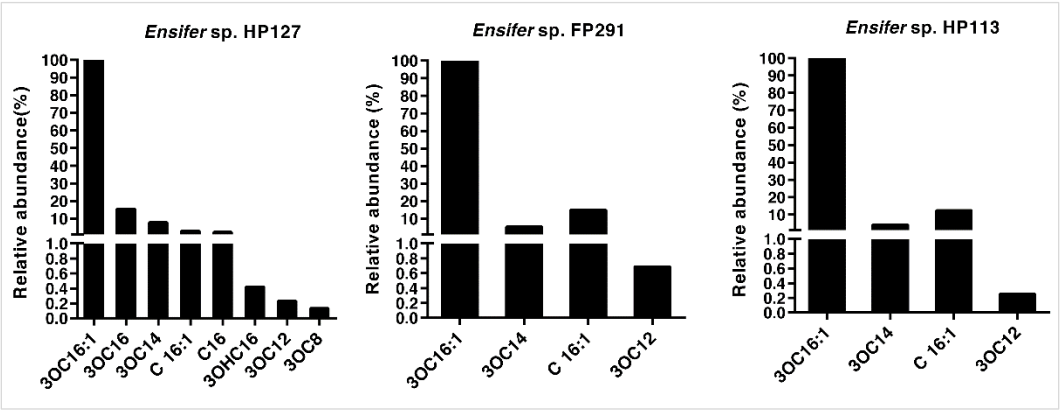


Figure 2.4 Semiquantitative estimations of the AHLs produced by each of the three isolates. X-axis lists the AHLs in the order of their relative abundance. The Relative abundance in % on y-axis is calculated based on the peak area returned by the MRM LC–MS/MS assigning 100 to the most abundant AHL.

2.3.5 Detection and sequence analysis of QS gene homologues of *Ensifer* isolates

Using the primers designed in this study (Table 2.2), homologues of *sinI, sinR* as well as *traI, traR* and the solo regulator *expR* were detected in all the three *Ensifer* strains (Figure 2.5). The PCR primers were designed to amplify nearly full length gene fragments. For *sinI, sinR, traI, traR* and *expR* the amplicon sizes were approximately 88%, 95%, 95%, 71% and 50% of their respective full-length reference homologue respectively. The amplified product of all the genes from all three isolates was sequenced and the DNA sequences submitted to NCBI GenBank database. The GenBank accession numbers are given in Table S1. NCBI BLAST analysis revealed that the QS genes in the *Ensifer* strains had significant sequence variability when compared to the QS genes sequences of well-characterized *Ensifer* strains– *E. meliloti* 1021 and *E. fredii* NGR234. Table 2.4 shows the sequence comparison matrix for these genes and homologues in characterized *Ensifer* sequences. The QS gene sequences of isolates differed with each other as well as with the reported *Ensifer* spp. While all of the chromosomal origin QS systems (*sinI, sinR and expR*) (González and Marketon, 2003) were relatively more similar to *E. meliloti* as compared to *E. fredii*, the plasmid-origin sequences (*traI* and *traR*) were found to be more similar to the latter than the former. Figure S1 of supplementary section shows the neighbour joining trees made to draw similarity comparisons among the isolates and the sequences annotated in other members of the *Ensifer* spp. InerproScan analysis predicted the presence of autoinducer synthase domain (member of Acyl-coA acyltransferase superfamily) in sinI and traI, and presence of AHL-binding domain as well as DNA binding domain with Helix-turn-Helix motif in traI and traR. Further, the protein sequence alignment revealed a heterogeneous pattern of distribution of sequence variation within different genes among the isolates. With the AHL synthases (*sinI* and *traI*), the sequence variations were found to be greater in abundance in the C-terminal region as compared to the rest of the sequence. In case of the *luxR* homologues (*sinR* and *traR*) the variations were found to be restricted to only the AHL binding domain and no variations were seen in the DNA binding domains. While for all the genes,

most of the amino acid variations observed were common among the three isolates, they were also found to harbor unique variations among themselves. The domain detection and variation mapping are summarized in Figure S2 of supplementary section.

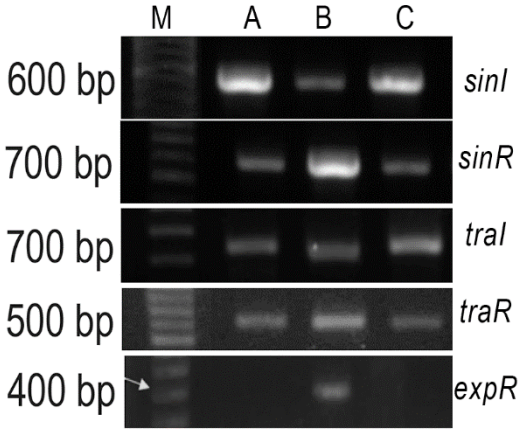


Figure 2.5 Detection of QS gene homologues from the *Ensifer* isolates. The bands show amplification of the QS genes (The gene name- labelled on the right side of the gel) from the total DNA of isolates: *Ensifer* sp. HP113 (A), HP127 (B) and FP291 (C). M denotes DNA size marker.

2.3.6 Regulation of Biofilm formation and chemotactic motility by quorum sensing in the *Ensifer* isolate HP127

Biofilm formation of *Ensifer* sp. HP127 on polystyrene microtiter plate was assessed after treating the low density cells (LDC) of the strain with its own AHL extract or synthetic AHLs. LDC were seen to not form significant biofilm after 24 h of incubation (Figure 2.6A). However, when treated with the AHL extract to, a significant increase in the biofilm formation was seen. Similar effect was seen when C16:1-HSL—the most abundant AHL produced by the isolate was used for induction instead of AHL extract. Vanillin (250 μgml^{-1}) was used as an inhibitor of QS mediated biofilm formation. Vanillin was found to prevent the formation of biofilm even in the presence of added AHLs indicating the increase in the biofilm was specifically QS-mediated. No significant difference in planktonic growth was

observed with any of the treatments as measured by an OD_{600} prior to staining for the biofilm, again indicating that the biofilm formation was enhanced and not the general growth.

Chemotaxis in rhizobia results into elicitation of flagellar motility in response to a chemical stimulus. LDC suspension of *Ensifer* sp. HP127 was observed to elicit positive chemotactic response to 1 mM proline which is known to be a potent positive chemoattractant in rhizobia (Gotz *et al.*, 1982) (Figure 2.6B). Upon addition of AHL extract or synthetic C16:1 to the cell reservoir, chemotactic response to proline was reduced but addition had little effect in the absence of proline. This indicated that QS was inhibiting the chemotactic motility.

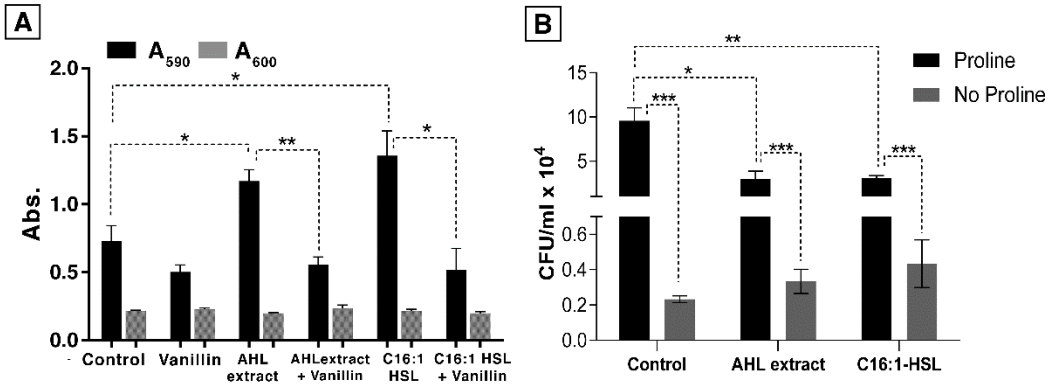


Figure 2.6 Assessment of regulation of biofilm formation and chemotaxis phenotypes in *Ensifer* sp. HP127. Y-axis in (A) represents absorbance at different wavelengths. A_{590} is the absorption by the crystal violet stained biofilms whereas the A_{600} represents the turbidometric assessment of growth before biofilm staining. In (B), Y-axis represents CFU/ml obtained from the syringe. In both cases, the error bars represent standard error of mean. n=3; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

QS gene homologue	Reference strain	Isolate strain								
		Ensifer sp. FP291			*Ensifer* sp.HP113			*Ensifer* sp. HP127		
		% QC	E-value	%Sim.	% QC	E-value	%Sim.	% QC	E-value	%Sim.
sinI	Em1021	100	6e-139	81	100	1e-138	82	99	2e-130	82
	EfNGR234	99	5e-143	83	100	9e-146	83	99	5e-130	81
sinR	Em1021	99	3e-148	80	100	2e-160	80	99	3e-157	80
	EfNGR234	99	7e-155	80	99	3e-153	78	100	2e-144	78
expR	Em8530	100	8e-121	89.73	100	1e-131	91.67	98	5e-119	90
	EfNGR234	100	7e-122	90.03	99	3e-132	91.94	99	3e-117	90
traI	EmRm41	59	2e-67	69	99	3e-31	69	98	2e-32	69
	EfNGR234	96	2e-75	83	100	6e-110	84	98	1e-105	84
traR	EmRm41	100	2e-169	82	99	2e-155	81	99	1e-164	82
	EfNGR234	100	0	87	99	0	88	99	0	87

Table 2.4 Sequence similarities of QS gene homologues detected in the pigeon pea nodule isolates and Ensifer strains characterize for QS. Em and Ef indicate *Ensifer meliloti* and *E. fredii* respectively; the digits following the letters are the strain names. Reference strain EM8530 was used for comparison since Em1021 carries insertion in the *expR* gene while for *traI* and *traR*, Rm41 was chosen as the reference as Em1021 does not possess these plasmid borne genes.

2.3.7 Transcriptional control of symbiotically crucial phenotypes by quorum sensing in the *Ensifer* isolate

Ensifer sp. HP127 was studied for the QS-mediated regulation of expression of genes that determine the symbiotic competence such as *flgD (flagellar biosynthesis), cheY1 (chemotaxis), pilA1 (formation of pilli), ndvA* (motility), *aglE* (utilization of alphaglucosides), *expE6* (EPSII biosynthesis). Additionally, the *sinI/R* gene expression was also studied, since they are known to be at the top of QS regulation and show the 'autoinduction'– quintessential of the QS in Gram negative bacteria. Figure 2.7 shows the fold change values of these genes when low cell density culture (LDC) was treated with AHL extract or the synthetic C16:1-HSL. High cell density culture (HDC) us used as a standard positive control since the QS mediated genes are regulated differently at higher cell densities as compared to the lower densities. Baseline in the plot represents magnitude of expression of the genes in LDC. Downregulation of varied magnitude was observed in the expression of *flgD, cheY1*, pilA1, *ndvA*, and *aglE* in the high cell density as well as when treated with AHL extract and pure C16:1-HSL as compared to the LDC. One of the crucial regulators of symbiotically important exopolysaccharides in *E. meliloti*, *expE6*, was upregulated by 12 to 16 -fold on AHL inductions. Likewise, *sinI* was significantly upregulated by 11 to 17 -fold on AHL treatments. Expression of *sinR* was moderately increased by about 2.8 to 3 –fold upon AHL induction however there was no significant difference in the expression between the LDC and the HDC (fold change < 2).

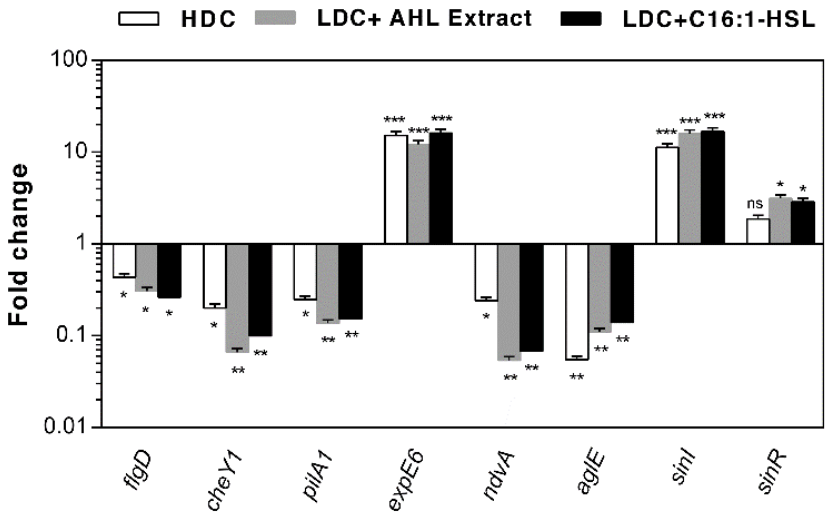


Figure 2.7 Gene Expression analysis of symbiotically important regulatory genes form *Ensifer* sp. HP127 upon induction by AHL extract (grey bars) and synthetic C16:1-HSL (black bars) using q-RT PCR. Y-axis indicates mean fold change values in the gene expression. The baseline represents the magnitude of expression of the respective genes in Low Density Cells (LDC) against which the other three sets are compared. Fold difference in the gene expression in HDC as compared to the LDC is indicated by white closed bars. Only the genes with fold change values ≥ 2 or ≤ 0.5 have been considered differentially expressed. Error bars indicate standard error of means. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n=3.

2.4 Discussion

Quorum sensing systems in rhizobia govern many of the cellular processes that are central to its symbiotic association with the. Many of these processes are conserved across different genera of rhizobia and yet, the QS in rhizobia is known to be versatile and have significant variation including that across species of the same genus or even members of the same species host (Calatrava-Morales *et al.*, 2018). This makes the study of QS imperative in a wider variety of rhizobia in order to understand their symbiotic physiology. This chapter dealt with characterization of the QS phenomenon in the rhizobia nodulating pigeon pea. Pigeon pea has been regarded as the poor man's meat (Gates, 2014) and the legume-crop of choice for small farmers (Varshney *et al.*, 2012) for its nutritional and agroeconomic

importance, especially to the global south. Three QS positive pigeon pea nodule bacteria were selected on the basis of efficient nodulation on as well as plant growth promotion of pigeon pea. They also possessed other PGPR activities such as siderophores and auxins production. Rhizobia, apart from nitrogen fixation, can perform host growth promotion and thus are better as biofertilizers, in fact, some of the rhizobial strains have been used with cereal crops as well (Naseer *et al.*, 2019; Matse *et al.*, 2020). The three isolate strains were identified to be *Ensifer* spp. (earlier known as *Sinorhizobium*), although pigeon pea has been reported to be nodulated predominantly by *Bradyrhizobium* along with *Rhizobium* and *Ensifer* spp. (Fossou *et al.*, 2016). Eight different AHL molecules were detected from the three *Ensifer* spp., all eight being produced by strain HP127 while the other two produced four each. Of the eight AHLs, six are reported to be produced by *E. meliloti* 1021 (Marketon *et al.*, 2002) while 3-O-C12-HSL and 3-OH-C16-HSL, have not been reported in *Rhizobiaceae* so far. The former has been widely reported in *Pseudomonas spp*. (Saraf *et al.*, 2014; Lee *et al.*, 2018) and the latter in *Serratia liquefaciens* (Cataldi *et al.*, 2008) and *Paracoccus* sp. (Saurav *et al.*, 2016), in both cases with highly sensitive MS/MS setups. Interestingly, 3-O-C16:1-HSL –the most abundant AHL produced by all the three *Ensifer* isolates in this study is also the highest produced AHL in *E. meliloti* 1021 (Gao *et al.*, 2005).

QS gene homologues of the rhizobial strains were detected by a set of highly specific degenerate PCR primers designed in this study for individual pairs of autoinducer synthase and corresponding response regulator gene. Homologues of *sinI*/*ngrI; sinR*/*ngrR*; *traI, traR* and *expR* were detected in all the three isolates. Homologues of s*inI* and *sinR* varied by 17- 20% from the reference sequences, however *expR* homologue was more conserved with only about 10% variability at the nucleotide level. This could be as a result of a very criucial role of *expR* in *Ensifer* by of regulating hundreds of promoters determining many of he processes responsible for survival in rhizosphere and nodulation process (Charoenpanich *et al.*, 2013). In reported *Ensifer* spp. *sinI,* and *sinR* homologues are chromosomally encoded, while the *traI* and *traR* genes are housed on the plasmids (Sanchez-Contreras *et al.*, 2007). While the sinI and sinR homologs detected in the isolates

are relatively more similar to Em1021, the *tra* loci detected in the isolates, were more similar to the *E. fredii* sequences than those from *E. meliloti*. This hints at a possible horizontal plasmid transfer phenomenon. *SinI* in *E. meliloti* synthesizes mainly long chain AHLs (Marketon *et al.*, 2002), and detection of *sinI* homologue and a large number of long chain AHLs in all the three pigeon pea isolates corroborate the same. Earlier reported primers of Huang *et al.*, (2013) for detection of autoinducer synthases from *Rhizobiaceae* amplified small gene fragments and were not designed to distinguish between different autoindicer synthases. The primers designed in this study give nearly full length amplification of the individual autoinducer synthases as well as response regulator genes. These primers may be of utility to screen for specific homologues from culturable populations as well as from metagenomic samples. Further, the detection of the canonical domains of luxI/R components confirm the findings. The variation in the amino acid residues in the SinI/R/TraI/R may be attributed to the production/binding of and to novel AHLs observed in the isolates, additionally, the possibility of promiscuity in the QS can also be an alternative (Gould *et al.*, 2006). The absence of any amino acid variation in the DNA binding motif is intuitive owing to the cruciality of the function,

Quorum sensing in rhizobia governs a number of cellular processes that collectively determine their fitness and fate (Wisniewski-Dyé and Downie, 2002). Among these, the most crucial processes for effective host-colonization are motility and attachment to the roots (Lopez-Garcia *et al.*, 2002). Additionally, since the higher cell densities of rhizobia are commonly found on the roots and they undergo a shift in the preferred carbon source, for which the genes regulating nutrient uptake have also been reported to be under QS (Hoang *et al.*, 2004; Gao *et al.*, 2005). In this work, upon induction by the AHLs, the genes promoting motility (*flgD, cheY1, pilA1, ndvA*) and utilization of alphaglucosides (*aglE*) were seen to be downregulated by QS, whereas the gene governing EPSII responsible for attachment (*expE6*) was upregulated by QS. The flagella capping protein, *flgD,* that assists in recruitment of new *flgE* subunits at the growing end of flagella (Moriya *et al.*, 2011) was seen to be downregulated by AHLs made by the *sinI* gene product

in *E. meliloti* and had a complex dependence on the *expR* gene (Hoang *et al.*, 2004). The present results are in agreement with the findings of Hoang *et al.* (2004) that in *Ensifer*, population density downregulates motility. *cheY1* regulates unidirectional flagellar movement in response to chemotactic stimuli (Sourjik and Schmitt, 1996) and has also been reported to be subject to similar QS regulatory scheme as *flgD* (Hoang *et al.*, 2008). *pilA1*, coding for the structural pilin subunit, in an earlier study (Hoang *et al.*, 2004), was found to be under a negative control of QS, and *pilA1* mutants were severely compromised in competitive nodulation. Although, the exact mechanism by which pilus affect the colonization is not known, it is postulated that Type IVb pilus must be of relatively greater importance during the initial attachment to the host root hairs (Zatakia *et al.*, 2014). The rhizobial *ndvA* is a homologue of *E. coli* hemolysin *hlyB*, and is involved in the secretion of cyclic glucans that regulate motility and affect nodulation capability (Stanfield *et al.*, 1988). Additionally, based on a recent observation that *ndvA* mutants were upregulated for EPS I production (Barnett and Long, 2017), it is likely that the repression of *ndvA* by QS (as found in this study) may lead to repression of local motility as well as promoting exopolysachharides production which will help in attachment and local spread. *aglE* is an α-glucoside transporter regulating uptake of sucrose, and additionally maltose and trehalose (Jensen *et al.*, 2002). While it was seen to be independent of QS in *E. meliloti* in a study by Gao *et al.* (2010), earlier proteomic studies from the same group observed *AglE* to be induced in QS mutants indicating a repressing effect of QS on its expression (Gao *et al.*, 2005). Additionally, since the disaccharides are dominant C-source in decaying material in the bulk soil (Jensen *et al.*, 2002) and that *Ensifer* spp. prefer dicarboxylic acids of the plant origin over sugars when in the rhizosphere (Iyer *et al.*, 2016), *aglE* downregulation may indicate switching to favorable carbon source on the roots.

One of the most crucial genes responsible for synthesis and secretion of galactoglucans (EPSII) is *expE6*. We observed over ten-fold upregulation in its expression on AHL induction. It was seen to be downregulated in *sinI* deficient mutants of *E. meliloti* (Gurich and González, 2009). Additionally, Gao *et al.* (2005) also observed a rescue of inhibition of *expE6* in QS mutants with externally added

AHLs. Also known as Exopolysaccharides II, these glucans are of remarkable significance for efficient nodulation (Pellock *et al.*, 2000). The upregulation of *expE6* gene on AHL induction in our isolate is consistent with the above-mentioned observations and indicates the promotion of attachment by QS in the isolate.

SinI and *sinR* are part of the master regulatory circuit of the QS in *E. meliloti*. *SinI*-synthesized AHLs drive the QS operation in the cell via regulatory proteins such as expR. *SinR* is indispensable for the upregulation of *sinI* and thus directly controls the production of AHLs. E*xpR* bound to these AHLs upregulates self and also binds to more than 30 different types of binding sites in the promoters of a large number of diverse genes and alters their expression in an AHL-concentration dependent manner (Calatrava-Morales *et al.*, 2018). On AHL induction, we obtained a sharp increase in *sinI* expression as well as a slight upregulation of *sinR expression*. The upregulation of *sinI* is likely to be the result of 'autoinduction' by long chain AHLs synthesized by itself as in *E. meliloti* 1021 (Teplitski *et al.*, 2003). Conversely, *sinR*, in a report by (McIntosh *et al.*, 2009) was found to be unaffected on AHL treatment of the wild type *E. meliloti* strain 2011 and was co-regulated by phosphate availability to the cells. Later, an AHL-concentration dependent repression of *SinR* was demonstrated in *E. meliloti* 2011 by Charoenpanich *et al.* (2013). However, in our experiments sinR was found marginally upregulated on AHL treatment as compared to the untreated LDC, while there was no significant change in the expression in HDC. Since the *sinR* expression is subject to intricate regulatory mechanisms involving a high concentrations of AHLs and variable binding attributes of the ExpR regulator in its promoter, it is very likely that the observed change in our study could arise from the strain differences, or the experimental conditions in addition— esp. given the reliance of the *sinR* expression on the nutritional status of the cell (McIntosh *et al.*, 2009; Charoenpanich *et al.*, 2013). A multi–time-point experimental setup may be required to explain the expression pattern of *sinR* in response to AHLs and nutritional conditions of the cells in our isolates.

Thus, the expression profile of the genes suggests that QS regulates motility and attachment in a reciprocal manner wherein high cell density leads to inhibition of motility and promotion of attachment in *Ensifer* sp. HP127. Such reciprocal regulation of attachment and motility is a widely observed phenomenon in other bacterial genera such as *Yersinia* (Atkinson *et al.*, 2006), *Pseudomonas* and *Vibrio* spp. as well as in *Ensifer* species including *E. meliloti* 1021 (Amaya-Gómez *et al.*, 2015) and *E. fredii* NGR234 (Krysciak *et al.*, 2014). In both the *Ensifer* species, this mutually inverse regulation is controlled by QS. Such a regulation makes evolutionary sense because on the root, active motility plays negative role for the dispersion and establishment of rhizobia whereas mucous secretion has been correlated with increased colonization (Caetano-Anollés *et al.*, 1992; Lopez-Garcia *et al.*, 2002). The repression of motility and promotion of attachment reflected in the gene expression analysis also confirm the observations of phenotypic assays. In spite of the difference in the genetic and chemical components as well as host plant species and geographic locations, a conserved regulatory scheme suggests evolutionary relationships between the *Ensifer* spp. To the best of our knowledge, this is the first report of quorum sensing in pigeon pea-nodulating *Ensifer* spp.