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Elucidation of quorum sensing components and their role in regulation of symbiotically important traits in *Ensifer* nodulating pigeon pea

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ABSTRACT

Quorum sensing (QS) in rhizobia regulates diverse processes determining the success and efficiency of association with the legume host. Despite the notable importance of QS as well as the well-known underlying variability in the genomic and metabolic components thereof, its study in rhizobia is largely restricted to few laboratory strains. In this work, QS phenomenon in the rhizobia nodulating pigeon pea– one of the most important legume crops of the global-south, is characterized. Using 16S rRNA and recombinaseA sequencing analysis, the selected QS-positive and host-beneficial isolates were identified to be taxonomically affiliated to the genus *Ensifer*. Their QS components, including homologues of QS genes, and the repertoire of N-acyl homoserine lactone (AHL) autoinducers were identified. Sequences of the QS homologues showed significant variabilities ranging from 10 to > 20% with the known *Ensifer* sequences. Autoinducer profiling using LC–MS/MS revealed the production of long and short chain AHLs variably by the isolates, including 3-oxo-C12-homoserine lactone (3-O-C12-HSL) and 3-OH-C16-HSL as their first report in *Rhizobiaceae*. Motility and attachment– two of the most crucial traits for effective establishment on host roots were discovered to be QS dependent in *in vitro* analysis and the same was confirmed using expression analysis of their regulatory genes using qRT-PCR; both revealing a QS mediated repression of motility and promotion of attachment. This study highlights that *Ensifer* nodulating pigeon pea, although with significant variance in the anatomy of their QS components, regulate symbiotically crucial cell-processes via QS in a scheme that is conserved in multiple genera.

1. Introduction

Pigeon pea (*Cajanus cajan*), a perennial legume of the family *Fabacae*, is cultivated in more than 20 countries and on close to 7 million hectares of land (Saxena et al., 2010; Chanda Venkata et al., 2018) and 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 is reported to be nodulated by many genera of rhizobia, notably, *Bradyrhizobium, Rhizobium* and *Ensifer* (earlier known as *Sinorizobium*) (Fossou et al., 2016). According to Herridge et al. (2008), pigeon pea obtains about 65% of its nitrogen from symbiotic nitrogen fixation by rhizobia thus making the latter a crucial subject of investigation. In most rhizobia, however, a large number of cellular 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 a mechanism of collective regulation of genes in bacteria in response to population density. The most important type of QS in Gram negative bacteria relies on synthesis, accumulation and detection of one or more types of autoinducer molecules, known as N-acyl homoserine lactones (AHLs), and is usually operated via pair(s) of luxI-type AHL synthases and luxR-type response regulators. These collectively, orchestrate the dynamic modulation of relevant cellular activities depending on the population density (Whitehead et al., 2001). Multiple *luxRI* type circuits are reported in rhizobia such as *sinRI* or *ngrRI* and *traRI* in *Ensifer* spp.; *cinRI, rhiRI, raiRI* and *traRI* in *Rhizobium* spp. and are often arranged in a functional hierarchy (Sanchez-Contreras et al., 2007). In *Ensifer* strains SinI is responsible for the production of diverse long-chain AHLs which regulate the expression of itself as well as several other promoters via *luxR* family transcriptional regulators such as ExpR. 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 coordinated and intricate 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

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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 are focused mainly on 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).

This study focuses on a multifaceted and detailed analysis of quorum sensing phenomenon of rhizobial isolates from the root nodules of *C. cajan*. Nodule occupants were screened for AHL production and host growth promotion. Genetic components of QS, chemical diversity of autoinducers, and regulation of symbiotically important phenotypes were characterized in the screened strains. Subsequently, the transcriptional control of genes regulating these traits was assessed. This study, thus, has generated a multicomponent anatomical and operational map of the QS phenomenon in *C. cajan* nodulants.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains used in this study have been enlisted in Table 1. The rhizobial isolates were routinely grown on Tryptone yeast extract (TY) medium (6 g/l tryptone type I, 3 g/l yeast extract) with added 3 mM $CaCl_2$ 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 some of the bioassays and was propagated in a manner similar to other rhizobial strains with supplementation of streptomycin (100 ug/ml).

2.2. Isolation of bacteria from pigeon pea nodules

Pigeon pea plants were collected from the agricultural fields around Vadodara, Gujarat, India. Nodules were plucked off the root carefully while not damaging their integrity and topically cleaned by a wash with 70% ethanol preceded and followed by washes with sterile distilled water. Surface sterilisation was done by swirling and gently vortexing in 0.1% mercuric chloride for 45 s, followed by five thorough washes with sterile distilled water, each for 30 s. Surface sterilized nodules were crushed in 50 µl of sterile Yeast extract mannitol (YEM) (Vincent, 1970) broth, 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.

2.3. Plant inoculation 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. Surface sterilized seeds of pigeon pea (Variety BDN-2, obtained from Pulse Research Station, Anand Agricultural University, Model Farm, Vadodara, India) were allowed to germinate for ~48 h in petri plates containing 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 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.

2.4. Biosensor based detection of N-acyl homoserine lactone production

Bacterial isolates were screened for production of AHL molecules by employing one of the most versatile AHL-biosensors, *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 in brief is as follows. The spent culture supernatants of strains 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 sought as a true positive result.

2.5. Taxonomic identification of bacterial strains

Bacterial isolates were identified taxonomically using sequences of 16S rRNA and *recA* genes. PCR amplification of the 16S rDNA was carried out from the isolated genomic DNA of isolates using 27 F and 1541R universal eubacterial primers (Pillai and Archana, 2008) and *recA* was amplified was 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).

2.6. Extraction of AHLs from bacterial culture supernatant

AHLs produced by the bacterial isolates were extracted employing liquid-liquid extraction of their late-log phase culture-supernatant with ethyl acetate as described in Shaw et al. (1997) with necessary modifications. 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. Supernatant was separated by centrifugation of the culture at 12,000 rpm for 10 min and extracted in 1:1 ratio twice with ethyl acetate pre-acidified with glacial acetic (0.2% v/v) acid. Ethyl acetate extract was subsequently evaporated using rotary evaporator

Table 1
Bacterial strains used in this study.

Bacterial strain	Relevant characteristics	Reference
Ensifer sp. FP291	Isolated from root nodules	This study
Ensifer sp. HP113	of pigeon pea, QS positive	This study
Ensifer sp. HP127		This study
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 González (2009)

(Rotavapor R-100, BUCHI, Switzerland) and 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. All the solvents used were HPLC grade and of Merck (USA) make.

2.7. 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 × 2.1, 3 µm RP column on Acquity® UPLC (Waters, USA). The parameters for liquid chromatography (LC) were standardized 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 – widely applied in AHL profiling, owing to its high sensitivity as well as the provision to return 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 N_2 was used as the collision gas. Six AHL standards (Sigma Aldrich, USA and Caymen chemicals, USA) were taken for validation (see Fig. S1D for the list of standards used).

2.8. Detection of quorum sensing gene homologues in bacterial 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 lists all the primer pairs, their annealing temperatures and expected amplicon sizes. PCR mixture contained, in addition to the buffer, 1.5 mM $MgCl_2$, 0.5 mM 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-30 s, T_a-45 s, 72 °C-1′; 72 °C-5′ (where T_a indicates annealing temperature which is specified for each primer pair in Table 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 sequences were submitted to NCBI GenBank for public availability. Supplementary Table S1 lists the accession numbers of these NCBI records.

2.9. Preparation of cells 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). The strain was first grown to OD_{600} of 0.5 in 5 ml TY broth in a tube. From this culture, the harvested and washed cells were used to inoculate fresh TY broth which was in turn grown to 0.2 OD. Similar passage was done subsequently to obtain a final culture suspension of 0.02 OD. 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 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 and would be used as one of the control sets for AHL induction experiments. For AHL induction, the LDC were incubated for 3 h based on Chen et al. (2003) with gentle shaking at 30 °C with either its own AHL-extract— prepared as described in Section 2.6, 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 (see Section 2.6 for preparation) 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.

2.10. Quantitative biofilm formation assay

Strains were tested for their biofilm formation capability on the polyvinyl chloride microtitre plate based on the method suggested by Fujishige et al. (2006) with following modifications. The cells were serially passaged to low density as described in Section 2.9. Each well in the plate contained 200 µl of LDCs. While untreated controls received dummy extract (Section 2.6), 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. OD_{600} was reported in order to assess the planktonic growth prior to staining the biofilms. The biofilms were subsequenty stained with crystal violet as desribed in the above mentioned report. Spectrophotometric plate reading was done using Tecan InfinitePro 2000 microtiter-plate reader. Each set was performed in triplicates and this was repeated thrice as individual experiments.

2.11. Chemotactic motility assay

Modified capillary assay was performed to investigate the role of QS in regulating chemotactic flagellar motility in the isolate 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 K_2HPO_4 + 3.9 mM KH_2PO_4) 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 2.6). 1 mM Proline, as originally suggested by Gotz et al. (1982) dissolved in chemotaxis buffer was used as the chemoattractant in the syringe ending 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 and compared across sets.

2.12. Real-time quantitative reverse transcription PCR

About 10^8 cells from each of the four sets prepared as in Section 2.9 were treated with 0.5 mg/ml lysozyme for exactly 5 min at 30 °C 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. 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 (1 µg) 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 chemisty. Oligonucleotide primers for qRT-PCR are listed in Table 2. The cycling conditions were as follows: 95 °C-100 s; 45 cycles of [95 °C-10 s, 60 °C-30 s, 72 °C-30 s]. 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

Table 2
Primer pairs used in this study for PCR-detection of gene circuits and qRT-PCR.

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

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.

expressed as fold change as given by Livak and Schmittgen (2001). Melting curve analysis was employed to verify absence of contaminating amplicons.

2.13. Statistical 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 equalling 0.05 were only considered as statistically signifcant.

3. Results

3.1. Isolation of nodule bacteria of Cajanus cajan and screening for host growth promotion and AHL production

Thirty bacterial isolates were obtained from the nodules of pigeon pea plants grown across five different fields. The strains were confirmed to be rhizobia by carrying out nodulation of pigeon pea plants in pot based plant experiments under gnotobiotic conditions. As seen in Fig. 1A, all except four isolates were able to develop nodules under experimental conditions while no nodulation occurred in the control where no rhizobia were inoculated. Among all the nodulating strains, five strains – FP272, FP291, HP113, HP127, and SA113 showed high nodulation (> 25 nodules per plant) indicating greater nodulation efficiency. The plant growth promotion as depicted by increase in plant weight and length (Fig. 1B and C) was substantial for strains FP291, HP113, HP114, HP123, HP125 and HP127. The bacterial strains were also screened for the production of quorum sensing signaling molecules as described in Section 2.4. As shown in Fig. 1D, the strains FP291, HP113 and HP127 showed distinct blue colored zone in the test plates and thus were considered as QS positive. Among the other isolates, AHL production could be detected in a few strains. Considering the efficient nodulation and plant growth promotion as well as proficiency at QS, the three nodule isolates FP291, HP113 and HP127 (marked by arrow heads in Fig. 1) were selected for further studies. FP291 was isolated from a farm in Fatepur, Vadodara, India (22°10′25.7″N, 73°13′20.2″E) while HP113 and HP127 were isolated from different plants from a farm in Hansapura, Vadodara, India (22°09′54.1″N 73°14′15.7″E).

3.2. Molecular phylogenetic identification of pigeon pea nodule isolates

The 16S rRNA gene fragments of strains FP291, HP113 and HP127 were PCR amplified and the sequences when analyzed by RDP-classifier identified all the three strains as belonging to the genus *Ensifer* with 100% confidence. All the three isolates showed over 99% similarity with *Ensifer terangae* as well as *E. mexicanus* and *E. chiapanecum* with EzTaxon. A neighbor-joining tree of 16S rRNA gene sequences (Fig. 2A) shows that the isolates cluster with *E. terangae*. In order to gain further insight, recombinaseA gene fragments of the strains were sequenced and phylogenetic tree generated from *recA* sequences (Fig. 2B) shows that the bacterial isolates are clades of *E. terangae*, *E. mexicanus* and *E. chiapanecum* branch. Thus, the strains FP291, HP113 and HP127 are denoted as *Ensifer* spp. and are taxonomically closest to *E. terangae.*

3.3. Chemical identification of AHL molecules secreted by pigeon pea nodule isolates

The chemical identities of AHLs produced by the *Ensifer* isolates were discerned by liquid chromatography tandem mass spectrometry (LC–MS/MS) with Multiple Reaction Monitoring (MRM). In order to

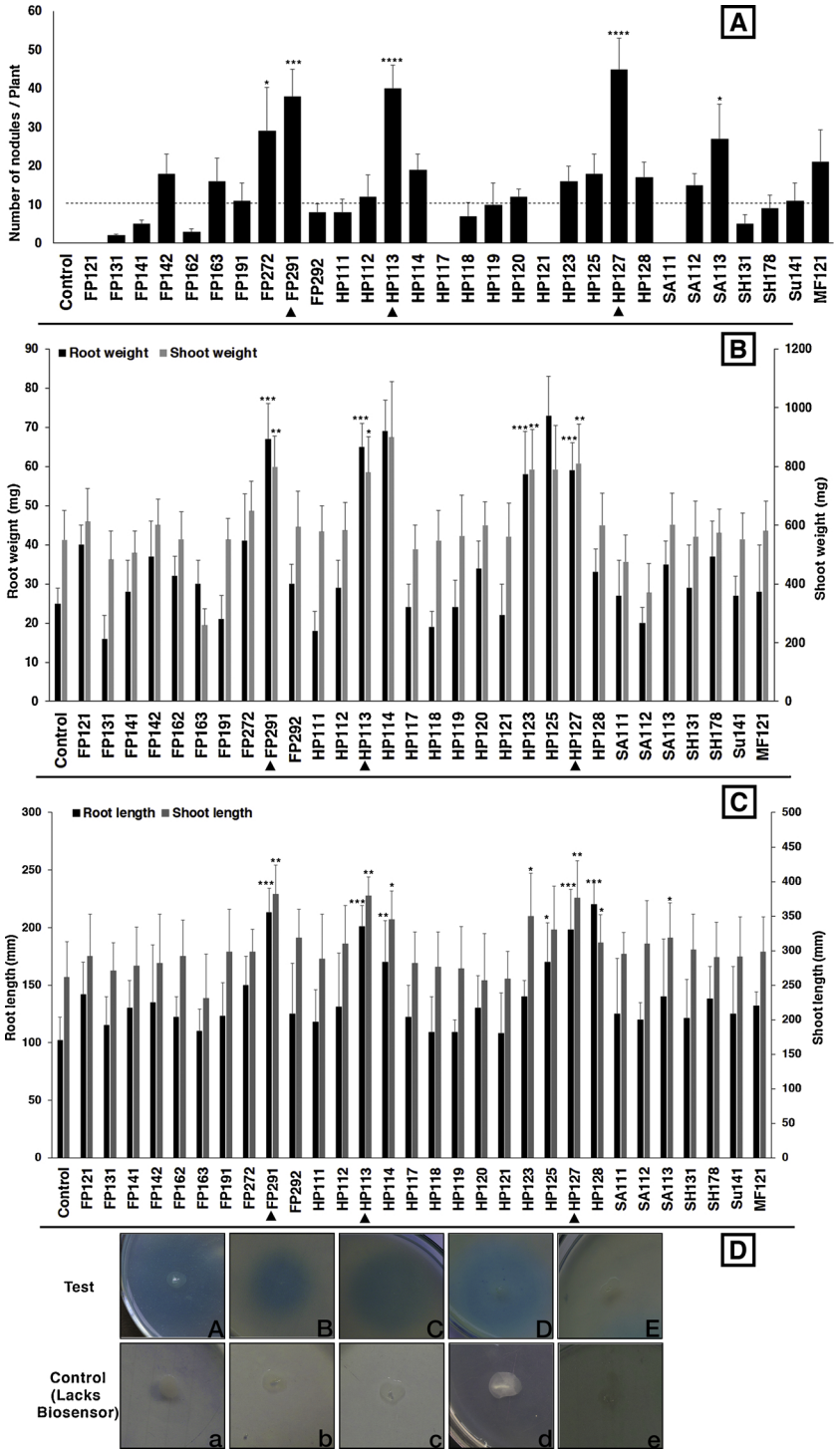

Fig. 1. Screening of pigeon pea nodulating bacteria for nodulation (A), plant growth promotion (B and C) via plant experiments and for production of N-acyl homoserine lactone signals (D) via biosensor mediated qualitative detection. The dashed horizontal line in Panel A represents mean of nodule numbers for all the strains. Panels A-C: 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 D: Upper row shows test AHL indicator plates for bacterial strains FP291 (A); HP113 (B); HP127 (C); *E. meliloti* 8530 (D) and AHL-synthesis-mutant of *A. tumefaciens* NTL4 (E). Bottom row (a–e) depicts the same strains on control plates that do not contain the indicator organism.

ascertain the identity of AHLs with confidence, we used the following four confirmatory conditions, only complying to *all* of which, was an AHL molecule reported to be present: (i) *m/z* of the detected parent fragment must match with the theoretical $[M+H]^+$ of 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 $[M+H\text{-}101]^+$, where M is the parental mass; if any of the two daughter ions were missing, the respective AHLs were 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, we were able to identify multiple AHLs in each of the isolates (Table 3). The mass spectra of AHLs produced by the isolates are given Fig. S1, in which the panels show peaks corresponding to each of the daughter ions for every AHL detected. Retention time and fragmentation patterns of the bacterially secreted AHLs matched with the appropriate standard. *Ensifer* sp. HP127 produced 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 Fig. 3. In all the isolates 3-oxo-C16:1-HSL was the highest

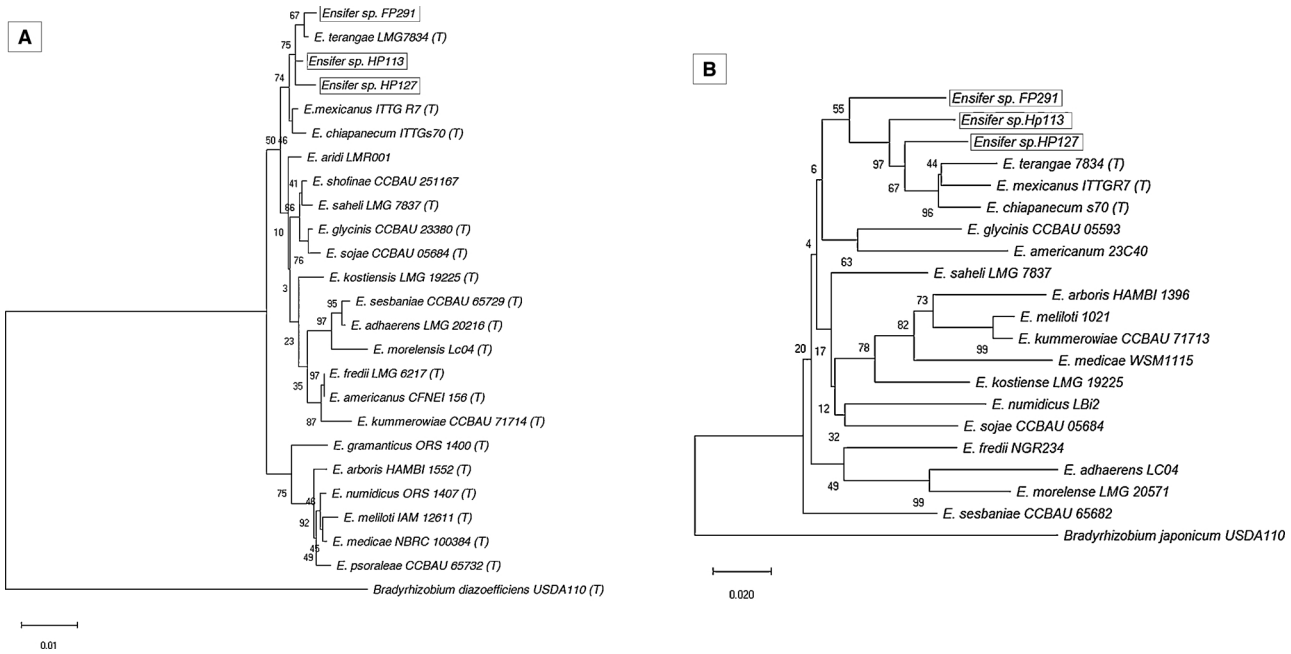

Fig. 2. Neighbour Joining tree using 16S sequences (A) and *recA* sequences (B) of *Ensifer* isolates of this study with related strains. Bootstrap values at nodes indicate average of 1000 replicates. 'T' in parenthesis indicates type strain. The tree is drawn to scale with evolutionary distances calculated using Kimura 2-parameter method. Isolates of this study are indicated by a box around their names.

produced AHL.

3.4. Detection and sequence analysis of QS gene homologues of Ensifer isolates

Using the primers designed in this study (Table 2), homologues of *sinI*, *sinR* as well as *traI*, *traR* and the solo regulator *expR* were detected in all the three *Ensifer* strains (Fig. 4). The PCR primers were designed to amplify nearly full length gene fragments (*sinI, sinR, traI, traR* and *expR* amplicon sizes were approximately 88%, 95%, 95%, 71% and 50% of their respective full-length reference homologue respectively). The DNA sequences of the amplified gene fragments were submitted to NCBI (GenBank Accession numbers are given in Table S1). NCBI BLAST analysis highlighted that the QS genes in the *Ensifer* strains bore significant sequence variability when compared to the QS genes sequences of well-characterized *Ensifer* strains– *E. meliloti* 1021 and *E. fredii* NGR234 (Table S2).

3.5. Effect of QS on Biofilm formation and chemotactic motility in Ensifer nodulating pigeon pea

Biofilm formation of *Ensifer* sp. HP127 on polystyrene microtiter plate was assessed under AHL induction. LDC were seen to not form significant biofilm after 24 h of incubation (Fig. 5A). In the set with added AHL extract to LDC, a significant increase in the biofilm formation was observed. Similar effect was seen when C16:1-HSL was added instead. Vanillin (250 $\mu g ml^{-1}$) was used as an inhibitor of QS mediated biofilm formation. Vanillin prevented the formation of biofilm even in the presence of added AHLs. 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 (Fig. 5A).

Chemotaxis in rhizobia results into elicitation of flagellar motility in response to a chemical stimulus. LDC suspension of *Ensifer* sp. HP127 showed a positive chemotactic response to 1 mM proline, known to be a potent positive chemoattractant in rhizobia (Gotz et al., 1982) (Fig. 5B). 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.

3.6. Transcriptional control of symbiotically crucial phenotypes by quorum sensing in the Ensifer nodulating pigeon pea

Ensifer sp. HP127 was studied for the QS-mediated regulation of expression of genes determining 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 since they are known to be at the top of QS regulation and show the 'auto-induction'– characteristic of the QS in Gram negative bacteria. Fig. 6 shows the fold change values for these genes when low cell density culture (LDC) was treated with AHL extract or the synthetic C16:1-HSL as well as in case of high cell density culture (HDC) as a standard positive control. 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 12–16 -fold on AHL inductions. Likewise, *sinI* was significantly upregulated by 11–17 -fold on AHL treatments. Expression of *sinR* was moderately increased by about 2.8–3 –fold upon AHL induction however there was no significant difference in the expression between the LDC and the HDC (fold change < 2).

4. Discussion

Given the underlying variability in the quorum sensing (QS) components and their operation, as well as the differences in downstream genes and QS-governed processes in rhizobia (Calatrava-Morales et al., 2018), it is imperative to study QS in a wider variety of strains. The present work characterized the QS phenomenon in the rhizobia nodulating pigeon pea- also known as the poor man's meat (Gates, 2014) and the legume-crop of choice for small farmers (Varshney et al., 2012). 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. The three isolate strains were identified to be *Ensifer* spp., although pigeon pea has been reported to be nodulated predominantly by

Table 3
N-acyl homoserine lactones detected from pigeon pea nodulating *Ensifer* spp. and their MS/MS transitions.

Strain	AHL	m/z of the Q1 parent	m/z of Q3 product ion $[M + H\text{-}101]^+$	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

Bradyrhizobium along with *Rhizobium* and *Ensifer* spp. (Fossou et al., 2016). However, since the AHL mediated QS is not prevalent in *Bradyrhizobium* spp. (Bogino et al., 2015), we may not have obtained them as QS positive in our selection scheme. 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., 2007) 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 pair 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 *sinI* 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 a repercussion of the substantial role *expR* plays in *Ensifer* by regulating hundreds of promoters (Charoenpanich et al., 2013). In reported *Ensifer* spp. *sinI,* and *sinR* homologues are chromosomally encoded, while the *tra* genes are housed on the plasmids (Sanchez-Contreras et al., 2007). The *tra* loci detected in our 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.

In rhizobia, QS regulates a multitude of 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 study 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

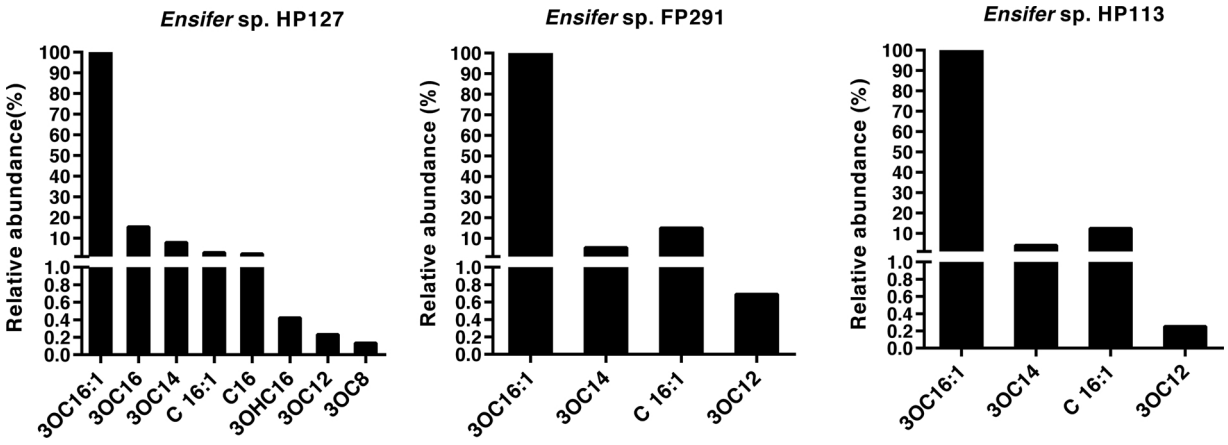

Fig. 3. Semiquantitative account of the AHLs produced by pigeon pea nodule isolates: X-axis lists the AHLs in the order of their relative abundance. The Relative abundance in % on y-axis is calculated based on the peak area returned by the MRM LC–MS/MS assigning 100 to the most abundant AHL.

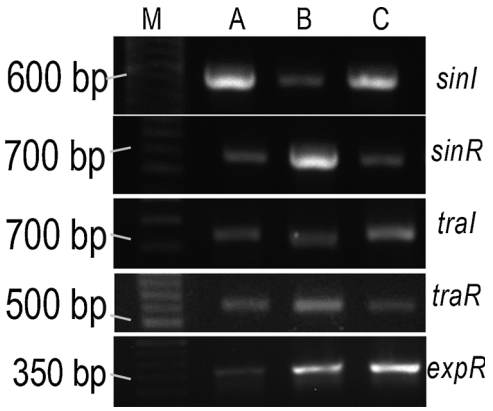

Fig. 4. Detection of QS gene homologues from pigeon pea nodule isolates: The bands show amplification of the QS genes (labelled on the right side of the gel) from the total DNA of isolates: *Ensifer* sp. HP113 (A), HP127 (B) and FP291 (C). M denotes DNA size marker.

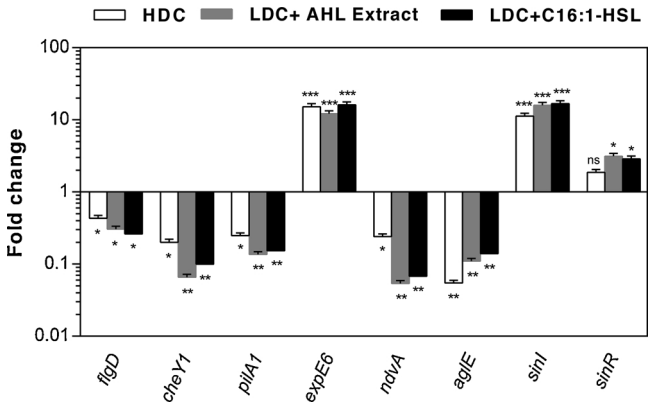

Fig. 6. Expression analysis of selected genes in *Ensifer* sp. HP127 upon induction by AHL extract and synthetic C16:1-HSL using q-RT PCR: The values on y-axis indicate mean fold change in the gene expression. The baseline represents magnitude of expression of the respective genes in Low Density Cells (LDC) against which the other three sets are compared. 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.

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

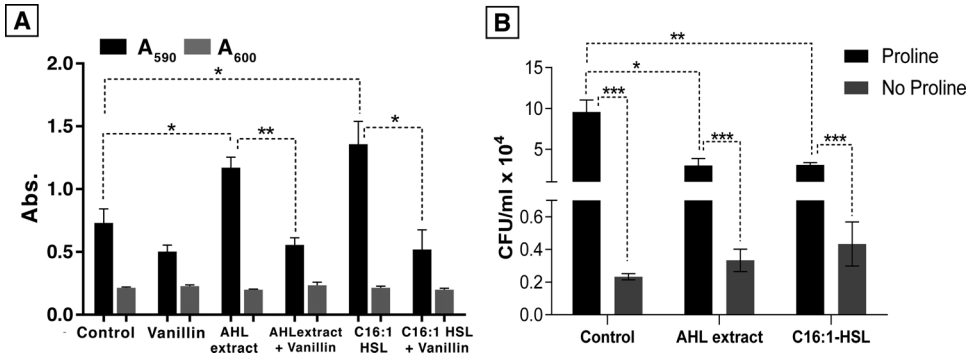

Fig. 5. Phenotypic assays of biofilm formation and chemotactic motility in *Ensifer* sp. HP127: Y-axis in (A) represents absorbance at different wavelengths. A_{590} is the absorption by the crystal violet stained biofilms whereas the A_{600} represents the turbidometric assessment of growth before biofilm staining. (B) Y-axis represents CFU/ml obtained from the syringe. In both cases, error bars indicate standard error of means. n = 3; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

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.

5. Conclusion

Exploration of the symbiotic physiology of pigeon pea symbionts (identified as *Ensifer* spp.) with respect to their quorum sensing revealed novel additions to the autoinducer molecules reported in *Rhizobiaceae* besides the known ones in well-studied *Ensifer* itself. Analysis of QS genes in the *Ensifer* isolates confirmed the presence of homologs of *sinI, sinR, expR, traI, and traR* in all the three pigeon pea nodule isolates. The strain *Ensifer* sp. HP127 was found to be responsive to AHL signals for a reciprocal regulation of formation biofilms and motility at phenotypic level. Expression of representative genes for flagellar motility, chemotaxis and production of EPSII were found to be under the regulation of quorum sensing in a manner similar to the reported strains of *E. meliloti* nodulating alfalafa. Thus, QS in pigeon pea nodulating *Ensifer* spp seems to be central to the establishment of successful association with host and indicates the conservation of mechanisms across rhizobia nodulating different host plant species.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.126354.

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