Synopsis of the thesis on

Approaches to Enhance the Nodulation Competitiveness of Rhizobia.

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By

Jitendrapuri Pratappuri Gosai

Department of Microbiology and Biotechnology Centre

Faculty of Science,

The Maharaja Sayajirao University of Baroda,

Vadodara.

Introduction

Rhizobia are Gram negative diazotrophic bacteria that form symbiotic association with legumes. In this association, they form specialised organs known as nodules on the legume-roots where the bacteria fix aerial N_2 to the reduced form, ammonia via a process known as Symbiotic Nitrogen Fixation (SNF) (Sugawara and Sadowsky, 2012). Most rhizobia belong to the Family *Rhizobiaceae* and majorly fall under 18 genera to occupy nearly 240 species. SNF globally accounts for at least 70 million metric tons of nitrogen per year with each hectare fixing up to 300 kg of N_2 (Brockwell et al., 1995; Peoples et al., 1995). The consequent recognition of rhizobia as bioinoculants to improve the productivity of legume crops led to their application as biofertilizers. However, the application has not been as productive as expected and the best results of rhizobial inoculations are obtained only when the soil has an absence or scarcity of the nodulating rhizobia. (Catroux et al., 2001). Meade et al., (1985) established that in case of *Rhizobium leguminosarum* inoculation, a minimum of 1000X cells of inoculant were required as compared to the indigenous strain numbers in order to achieve about 70% nodule occupancy by the inoculant. Similar observations have been made in case of *Bradyrhizobium* spp. (Pinochet et al., 1993) indicating that the practice of rhizobial bioinoculation has been a challenge due to the resident rhizobia in soil being more aggressive in colonization and subsequent nodulation. This underlying cause of inadequacy in effectivity of the introduced rhizobia is generally referred to as Nodulation competitiveness (NC) and can be defined as the superiority in the ability of a rhizobial strain, as compared to other virulent rhizobia, to nodulate a receptive legume host (Archana, 2010). One of the toughest hurdles in managing the competition problem is the lack of thorough understanding of the specific junctures during the root colonisation when inoculants are outcompeted by the indigenous strains (Sessitsch et al., 2002). Research in this regard has unraveled several biotic and abiotic factors that affect NC, nonetheless, the understanding has not resulted in a concrete solution to the problem due to the multiplicity of factors. Among the abiotic factors, various edaphic attributes such as the pH, temperature, consistency, salinity and nutritional status are the most important. The most common biotic factors that affect the survival and colonization of rhizobia are chemotaxis and motility, cell surface components such as exopolysaccharides, ability to use certain substrates such as rhizopines, synthesis of storage polymers, production of antimicrobial com- pounds, rates of growth and infection etc. (Archana, 2010; Sessitsch et al., 2002). Interestingly, many of these phenotypes are regulated as a matter of cell population densities by a phenomenon called as quorum sensing. (Sanchez-Contreras et al., 2007)

Quorum sensing (QS) is a mechanism of gene regulation in bacteria in response to the change in the population density. It occurs in diverse taxa of both the gram varieties of bacteria with common and unique features among them. A density dependent accumulation of an autoinducer- the signaling molecule, and detection of its threshold concentration subsequently, drive the regulatory phenomenon. Autoinducers in gram negative bacteria are different variants of amphipathic n-acyl homoserine lactone (AHL) differing in the fatty acyl chain in terms of its length, saturations and substitutions. A given strain may produce one or more of AHL molecules. A quintessential Gram negative quorum sensing system comprises of a pair of coordinately regulated pair of genes: the *luxI* homologue encoding AHL synthase, and a *luxR* homologue encoding a cognate receptor that binds to the AHL molecule and is a transcriptional regulator protein. A strain may have one or more of these quorum sensing circuits and in case

of multiplicity, they are often arranged in a regulatory hierarchy (Waters and Bassler, 2005; Whitehead et al., 2001). Most rhizobia harbor more than one QS circuits and produce multiple AHLs with significant variations among the different species. QS-regulated phenotypes encompass many of traits that regulate various symbiotically important processes (Sanchez-Contreras et al., 2007). This makes Quorum sensing an interesting avenue to explore into in order to engineer rhizobia to be symbiotically more competent.

Pigeon pea (*Cajanus cajan*) is a perennial legume of the family *Fabacae*. It is cultivated across more than 20 countries and on nearly 4.8 million hectares of land. (Saxena, 2010). It is very nutritive in nature with the dry seeds containing about 25% protein by weight. It has been regarded as the most important grain legume crop of rain-fed semi-arid tropics (Pal et al., 2011). India is the leading producer of this pulse and has it as a part of the staple diet in most parts of its geography. These aspects make pigeon pea a crop that demands more attention for its better yields.

Root is a dynamic substratum, continuously changing in its anatomy, physiology and biochemistry (Parke, 1991). It is the part of plant that exposes it to possibly the most diverse and numerous interkingdom relationships. These interactions under the soil determine the homeostasis and health of the plant at large. Legume-rhizobium interaction in the soil is remarkably complex and ultimately leads to attraction and colonization of the rhizobia on the roots where they subsequently engage into specific interactions with the root tissue to initiate the infection thread and establish nodules (Gibson et al., 2008). While a lot is known about the chemistry and physiology of rhizobial attraction to the roots and the molecular events leading to establishment of nodules, significant details about the growth and movement dynamics of rhizobia once attached to the roots in soil, remain undiscovered (Benizri et al., 2001; Parke, 1991). While these details may greatly add to the pool of knowledge of the symbiosis and deepen our understanding of the molecular details of the interaction between a soil-origin nodulant rhizobium and the host root, they are far more valuable for the rhizobial strains applied as inoculants (Benizri et al., 2001). Various factors of the host. A broader understanding of the root colonization patterns under controlled factors can aid in addressing the issue of nodulation competitiveness.

Rationale of the present study

Rhizobia regulate many of the phenotypes important for the nodulation as a function of their cell density by quorum sensing. While QS occurs generally in rhizobia and the mechanisms are fairly conserved, there are significant chemical, mechanistic and regulatory differences in operation of QS in different strains of rhizobia. QS phenomenon in the pigeon pea rhizobia is not well studied. Also, the effects of over expression of QS on the nodulation competitiveness of rhizobial type strains is not studied. Since, the root colonization by rhizobia applied as inoculant is crucial to their symbiotic competence, studying root colonization dynamics of rhizobia in space and time as a function of relevant factors is important. Expansion of knowledge about the pigeon pea rhizobia and their interactions with the host will not only contribute to a detailed understanding of their symbiosis but will also allow development of more productive bioinoculant tools for this important legume.

Objectives

1. To characterize quorum sensing (QS) in rhizobia associated with pigeon-pea nodules.
2. Engineer QS system of rhizobia and assess its effect on nodulation competitiveness.
3. To study root colonization dynamics of selected rhizobial bioinoculants

Results and discussion

Objective 1: To characterize quorum sensing in rhizobia associated with pigeon-pea.

Pigeon pea nodulating rhizobia were isolated from various fields in and around Vadodara. Sites included the fields at the villages- Hansapura, Fatehpura, Sundarpura, Shahpura and Salat as well as the field of Model farm, Pulse Research Centre, Anand agricultural University, Vadodara to obtain a total of 40 isolates. The isolates were subjected to qualitative determination of acyl homoserine lactone, the autoinducer produced and secreted by rhizobia for quorum sensing. This was performed via a plate based biosensor assay employing *Agrobacterium tumefaciens* NTL4(pZLR4) which does not produce AHL of its own and produces β-galactosidase in response to exogenously supplied AHLs which can be assayed by using chromogenic substrate such as X-gal (Shaw et al., 1997) . Ten of 40 isolates screened produced detectable levels of AHLs with the biosensor assay. These were subsequently subjected to further analysis and screening. These selected isolates were tested for their biofilm formation capabilities on the polyvinyl chloride plates (Fujishige et al., 2006) and were all found to form strong biofilms with variable intensities. They were also tested for their swarming motility (Tambalo, Yost, et al., 2010) on the semisolid agar medium and only about 50% of the isolates showed the presence of swarming motility. Further, 16S rRNA gene was amplified from the selected isolates and were sequenced. Based on the similarity with the entries in database, they were deduced to belong to the either of the *Rhizobium*, *Ensifer* or *Bradyrhizobium* genera. AHLs were extracted using liquid-liquid extraction in ethyl acetate from the spent culture medium of the isolates that produced the maximum amount of AHLs. The isolates used for further analysis were FP291, HP113 and HP127 which all belong to *Ensifer*. Rhizobia are known to produce multiple AHLs, so the isolates were tested for the presence of multiple AHLs. Ethyl acetate extracts dissolved in methanol were subjected to thin layer chromatography using 60:40 methanol:water as mobile phase to resolve multiple AHLs present (Shaw et al., 1997). All the isolates produced short and long chain length AHLs characteristic of rhizobia (Sanchez-Contreras et al., 2007). Ethyl acetate extracts were subjected to Liquid chromatography-tandom mass spectrometry (Gould et al., 2006) to learn the chemical identities of the produced AHLs. The following AHLs were detected to be present in HP127: 3-oxo-C8 HSL, 3-oxo-C12 HSL, 3-oxo C14 HSL, C16 HSL, 3-oxo-C16 HSL, 3-oxo-C16:1 HSL and 3-OH-C16 HSL. AHLs produced by FP291 are as follows: 3-hydroxy-C6-HSL, 3-hydroxy-C8-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL, C16:1-HSL and 3-oxo-C16:1-HSL. The two isolates, although taxonomically identical, possess unique as well as overlapping set of AHLs. These multiple AHLs are synthesized via hierarchically arranged multiple genetic circuits of *luxI-luxR* homologs in rhizobia; to identify the genetic circuits of AHL synthesis and detection, degenerate primers were designed for *sinR*, *sinI*, *traR*, *traI* and

expR. With these primers specific for the respective genes, the isolates were tested for their presence. The amplicons were sequenced to ascertain the identity. All the three isolates showed presence of *sinR* and *traRI* circuits, whereas *sinI* could not be detected in HP127N. *expR* is an orphan receptor with no cognate AHL synthase *luxI* homolog (González and Marketon, 2003). This was present in HP127N whereas it could not be detected in the rest of the two. This could mean an absence or the difference in the sequence of the gene, as in *S. meliloti* strain 1021 where it is inactivated by an insertion (Marketon and Gonzalez, 2002). Absence of *sinI* in HP127N is more likely to be due to the non-matching sequence given the fact that HP127 produces long chain AHLs which in other rhizobia are attribute to *sinI* (Marketon et al., 2002). To investigate the phenotypes controlled by quorum sensing in the isolates, q-RT PCR was performed. Genes known to regulate the phenotypes crucial to symbiosis such as motility, chemotaxis, adhesion to the roots and utilization of some carbon sources (Rinaudi-Marron and Gonzalez, 2013) were assessed for the differential expression profile in the low culture density and the same on induction by its own AHLs. The following genes were found to be differentially regulated. *aglE*, *cheY1*, *flgD*, *ndvA*, *pilA1*, *sinI* and *sinR*. High cell density culture was taken as a positive control. *sinI* and *sinR* were found to be upregulated by the AHL treatment as expected and validated the induction. *flgD* was downregulated about two fold, which is not as drastic as the other genes which were significantly downregulated to close to ten folds upon the induction by AHLs. The pattern of the expression suggested that the quorum sensing in HP127 inhibits chemotaxis and motility. This is in accordance with reports that suggest QS promotes adhesion on the roots once the high cell density is reached and as adhesion and motility are generally regarded as mutually exclusive and inversely regulated phenotypes (Amaya-Gómez et al., 2015). The expression profiles were also tested by the induction with chemically synthesized AHLs, including those that are produced by the isolate and one that is not. The induction of the phenotypes was observed to be specific by the AHLs that are produced the isolates. Since biofilm formation is an outcome of coordinated actions to colonize and adhere to a surface, it was tested to be regulated by AHL induction. There was a significant increase in the biofilm formation in 24 hours under static condition when the AHL was added to the low density cells as compared to when it was omitted; vanillin, a reported inhibitor of quorum sensing and biofilm formation (Kalia, 2013), quenched the induction of biofilm formation by AHLs confirming the cause-effect relationship. The three isolates are potent PGPR, too and upon inoculation to the host, they contribute significantly to the plant health. In order to potentiate their role as strong PGPR, important traits were studies. The obtained results are as follows. All the three isolates produce Indole acetic acid ranging from 9 to 14 µg per ml; while FP291 did not produce detectable levels of siderophores, as tested on CAS plates (Schwyn and Neilands, 1987), FP291 produced the maximum amounts while HP127 made the least amount of siderophores. HP113 and HP127 also lead to increase in the plant nitrogen and chlorophyll content. These isolates increased the plant dry biomass significantly as compared to uninoculated control in the unfertilized soil at the pot level and made the maximum number of nodules as compared to the rest of the isolates. Overall, this study explored the quorum sensing machinery and operation in the *Ensifer* isolates of pigeon pea and established them as potent PGPR and nodulators.

Objective 2: Engineer QS system of rhizobia and assess its effect on nodulation competitiveness.

Rhizobium leguminosarum strain 3841 and *Ensifer meliloti* strain 8530 were used for this study. These are type strains, with sequenced genome, well characterized physiology and known quorum sensing regulations. *Rlv*3841 nodulates pea, vetches and lentils whereas *Sm*8530 nodulates alfalfa (González et al., 2003). *Rlv*3841 and *Sm*8530 have *cinRI* and *sinRI* as the chief quorum sensing systems respectively, at the top of the hierarchy regulating the target genes and other quorum sensing circuits (Edwards et al., 2009; Gao et al., 2005). *cinRI* was cloned in the pBBR1MCS2 giving rise to pCINRI of ~6.6 kb. The vector confers resistance to Kanamycin. The construct was transformed in *E. coli* DH5α and confirmed using Restriction digestion analysis. The construct was subsequently transformed in *Rlv*3841 via electroporation and the clones were confirmed again by plasmid digestion by specific restriction digestion analysis. The transformant was studied for its AHL production dynamics. It was seen that the transformant produced AHLs since the beginning of the growth unlike the wild type that only produces detectable amounts of AHL upon high cell densities in the late log phase. The transformant also made overall increased amounts of AHLs across all phases of growth as measured on the *A. tumefaciens* biosensor plate assay. Quorum sensing in *Rlv*3841 promotes attachment and inhibits motility(Tambalo et al., 2010). Semiquantitative reverse transcription PCR analysis revealed a gene expression profile indicative of promoting adhesion and colonization. Exopolysaccharide production and biofilm formation play determinative role in the adhesion and subsequent colonization of the host root . Assays of Exopolysaccharides secretion revealed that it was significantly increased in case of the compared with the WT. The same was observed with the biofilm formation in the transformant while the upregulation was reversed by the addition of vanillin indicating that it was a specific effect of the QS upregulation. Swarming motility on the semisolid agar plates containing either of the glucose, mannitol, mannose, and maltose was observed to be decreased significantly in case of transformant as compared to that in the WT. This trend indicates that the QS overexpression promotes the adhesion and colonization related phenotypes while downregulating the phenotypes that are more relevant prior to coming in contact with the roots such as motility and chemotaxis. The transformant did not show enhanced plant growth promotion however that does not necessarily corelate with the nodulation competitiveness. *sinRI* was amplified from *Sm*8530 and cloned in pBBR1MCS5 that confers resistance to gentamycin. The construct was confirmed using restriction digestion analysis and was subsequently transformed in the *Sm*8530 in order to achieve homologous overexpression. The transformant was observed to have a constitutively operational quorum sensing untethered to the cell-density mediated regulation similar to the *cinRI* overexpression in *Rlv*3841. This study indicates that homologous overexpression of the master regulator QS system puts the rhizobium in an “always-on” QS state. This leads to an increased ability of the bacteria to attach to substratum while secreting more exopolysaccharides. This is of remarkable value for a rhizobium in case of application as a bioinoculant coated on the seeds before sawing.

Objective 3: To study root colonization dynamics of selected rhizobial bioinoculants

The reason for poor understanding of the challenged nodulation competitiveness of the inoculants partly stems from the fact that there is little knowledge of when exactly, during the process of colonization, the rhizobium faces the harshest competition by the autochthonous flora. The scenario can be improved by a more comprehensive understanding of how, upon inoculation of rhizobia they colonize on the roots of the host in the soil. In this study, the potent nodulators and strong PGPR strains of *Ensifer* nodulating pigeon pea were used. They were tagged with the fluorescent protein marker *eGFP* being expressed from the vector pBBR1MCS2. Tagged strains were subsequently coated on the germinated seeds which were then sawn in the soil. The constructs have a stable constitutive expression of *eGFP*. HP127 tagged with *eGFP* was assessed for the ability to sustain the plasmid without the selective antibiotic force *in situ*. 100% of the bacteria reisolated from all the parts of the root as well as the surface sterilized nodules sustained the plasmid in them until the period they were tested for i.e. five weeks. This allows the usage of the tagged isolated for the *in situ* application lasting until the nodulation period of the host, pigeon pea. Colonization study involving HP127 on the roots of pigeon pea in order to track the movement of the bacterium in space and time for a period of five weeks was undertaken. Germinated seeds of the pigeon pea were coated with high inoculum of HP127 of the late log phase, after an incubation of four hours, the roots were cut into three segments of 1 cm each to assess the homogeneity of the initial attachment and it was observed that the identical numbers of rhizobia attached on all the parts of the roots. One week later, the coated rhizobium had spread to the length of the roots of 6-9 cm. Maximum colonization was seen at the site of inoculation, i,e the proximal region to the seed coat and on the primary roots. Colonization was reduced with the secondary roots and as the distance from the seed coat increased. 1-4 x 10^5 rhizobia per cm of the primary root tissue of the proximal end, this increased linearly upto 1-8 x 10^8 by the end of five weeks. 3 cm from the seed coat the rhizobia increased from 1-4 x 10^4 to at the end of 1 week to 1-6 x 10^6 per cm of the root tissue. Thin tertiary roots on the distal ends variably contained from zero to upto 40 rhizobia per cm of the thin root after one week of the inoculation while increased to a variable number of zero to 1-3000 per cm of the thin root after five weeks. Tiny nodules started to appear after three weeks but they were few in numbers and nonspecific in the location. Nodule counts after the five weeks reveled the following. While 47-55% of the nodules formed on the top third of the root length, the mid third of the root had an average of 23-44% nodules and the distal thin roots had 0-15% of the total nodules formed. The nodules in the top regions were often larger as compared to the ones at the distal region indicating that the former probably arose earlier than the later. All the experiments were done in the same soil and the plants were watered manually in the same manner in all the cases to avoid the variation due to edaphic attributes such as pH or water activity as well as the movement of rhizobia caused by the flow of water during watering. Further investigation involving assessment of the effect of various factors such as method of inoculation and co-inoculation with another PGPR strains are currently under commencement. The pattern of the colonization indicates that the rhizobia are steadily increasing in the numbers at the site of their attachment. It is believed that the elongation rate of the root is remarkably faster than that of the division of rhizobia, hence they fall short of colonizing the growing root in real time; further, the motility is also reported to be not useful in traversing large distances (Caetano-Anollés et al., 1992) such as ~ 19 cm after five weeks. The factors relevant to this study that contribute to the movement of rhizobia on the root include

the strength of attachment of rhizobia to the root and the force of percolating water. The observed pattern of colonization seems to be in accord with the above knowledge, however, the fact that the isolate colonizes the farther root and nodulates the actively growing parts in the distal thin roots indicates the potential of the isolate as a bioinoculant while more knowledge needs to be generated about augmenting the colonization on the host roots.

Conclusions

Nodule occupants were isolated from the pigeon pea. They were identified to belong to either of the *Rhizobium*, *Ensifer* or *Bradyrhizobium* genera. The isolates did a growth promotion of the host in pots and were also tested positive for various PGPR traits such as Siderophores and indole acetic acid production in vitro. Isolates exhibited symbiotically important phenotypes such as biofilm formation and swarming motility. QS loci of the selected isolates were identified to reveal the presence of canonical luxRI circuits and some orphan AHL receptor varyingly. Profiling of the autoinducer AHLs from these isolates using LC-MS/MS revealed the presence and chemical identity of multiple long and short chain AHLs. Phenotypes governing the colonization on the roots and the motility of the isolate *Ensifer* sp. HP127 were revealed to be under the control of QS as studied by self-AHL induction followed by qRT-PCR, while these were also confirmed with phenotypic assays.

Homologous overexpression of the respective chief QS circuit was achieved in two type strains viz *R. leguminosarum* 3841 and *E. meliloti* 8530. Their quorum sensing was observed to have been uncoupled of the cell density mediated regulation and was constitutive in nature. The Rlv3841 transformant had an gene expression profile indicative of induced attachment phenomena and reduced motility as compared to the WT. These results were confirmed extended by the in vitro phenotypic assays.

Efficient nodulant and potent PGPR of pigeon pea *Ensifer* sp. HP127 was tagged with eGFP to investigate its colonization pattern on the growing root in the soil. There was no plasmid loss for the five weeks that it was monitored for. HP127 was observed to colonize all the lengths of the primary root as well branches and the thin roots with a variable tendency. The rhizobium did not only colonize the various part of the roots but also grew in numbers at the respective sites. Nodulation was observed to follow a similar pattern as the colonization.

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