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Effect of cell density and mutation on the expression of *Rhi* genes in *Rhizobium leguminosarum* biovar viciae

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ABSTRACT

Effects of cell density, symbiotic plasmid and mutation (by transposon mutagenesis) on the expression of *rhi* genes in *Rhizobium leguminosarum* biovar viciae were studied. Strains of the bacterium bearing and lacking regulatory gene *rhi*R were grown to late exponential phase and assayed for the production of *rhi* genes inducer. *R. leguminosarum* biovar viciae was specific for the synthesis of the *rhi* genes inducer. Expression of *rhi* genes advanced with increased cell population without any difference between Sym minus and Sym plus plasmid strains of the bacterium. Highest amount of the inducer was produced in the exponential phase. The growth of the microbe with and without Sym plasmid showed that symbiotic plasmid enhanced the optimal formation of *rhi* genes inducer. Iniducer formation in the absence of Sym plasmid was less than 20 Miller units of b-galactosidase activity in contrast to 2375 Miller units for plasmid-containing strain. Tn-5 mutagenesis generated four groups of mutants. Classes I, II, III and IV mutants were low, moderate-, high- and super-producers of the inducer. These groups showed 145-250, 625-896, 1031-1375 and 1563 Miller units of phosphatase activity respectively.

Keywords: Cell Density, Mutational effect, *rhi* genes.

INTRODUCTION

Rhizobium leguminosarum biovar viciae is a rod-shaped, motile, gram-negative, nitrogen-fixing bacterium which fixes nitrogen on legumes such as lentils, peas and vetches[1,2]. These legumes like others are important in the provision of body-building minerals, calcium, vitamins A and B, thiamine, niacin and oil[3-7].

Various research studies including works on *rhi* (rhizosphere-expressed) genes are being carried out on legume-rhizobia interaction to improve yields. These genes form an operon comprising of *rhi*ABC. The operon is located adjacent to *rhi*R on symbiotic plasmid of *R. leguminosarum*_biovar_viciae[8]. Rhizosphere-expressed genes are under the influence of *rhi*R. The *rhi*R is important in root nodulation. According to Gao *et al*.[9], the quorum-sensing system in a plant bacterium, *Mesorhizobium huakuii* affects growth rate and symbiotic nodulation. The *rhi* R encodes RhiR, a regulatory protein which functions as a transcriptional activator. RhiR has a homology with LuxR which belongs to LuxR-LuxI family. This class of transcriptional activators is common to a diverse group of gram-negative bacteria[10,11] including the quorum-sensing signal molecules and the LuxRI homologs that were identified in fish pathogen *Edwardsiella tarda*[12]. Comprehensive profiling of N-acylhomoserine lactones formed by *Yersinia pseudotuberculosis* using liquid chromatography coupled to hybrid quadrupole–linear ion trap mass spectrometry showed that the microbe belong to this group of transcriptional activators producers[13]. *Pseudomonas aeruginosa* possesses LasR which functions to activate virulence. *Agrobacterium tumefaciens* synthesizes TraR which is responsible

for conjugation control[14-17]. Also, *Agrobacterium vitis* bears a *luxR* homolog, *aviR*, that is associated with induction of necrosis on Grape and a hypersensitive response on Tobacco[18]. LuxR family protein SpnR which functions as a negative regulator of N-acylhomoserine lactone-dependent quorum sensing is present in *Serratia marcescens*[19]. LuxR in *Vibrio fischeri* regulates the expression of an operon *lux*ICDABEG in the presence of VAI (*Vibrio* autoinducer, N-3-(oxohexanonyl) homoserine lactone) thus influences luminescence in the microbe. LuxR-LuxI family is a population density-dependent gene activators.

The regulatory mechanism exhibited by LuxR-LuxI family is known as quorum sensing. It involves the interaction of self-produced extracellular signal compounds (autoinducers) with transcriptional activator proteins. In quorum sensing, after an adequate cell density is obtained, autoinducer normally accumulates which enables the bacterium to take a decision to regulate the expression of specific sets of genes[10,20]. Based on the homology which exists between RhiR and LuxR family, it was decided that investigation should be carried out on the principle of quorum sensing in *R. leguminosarum*_bv._viciae. In order to do this, we studied the effects of cell density and mutation on the expression of *rhi* genes in the bacterium. This could give an indication to the regulatory circuits involved in the symbiotic properties of the *R. leguminosarum*_bv._viciae.

MATERIAL AND METHODS

Organism and growth media

*Rhizobium leguminosarum*_biovar_viciae was obtained from American Type Culture Collection (Rockville, Md.). *Rhizobium meliloti* was kindly provided by Allan Downie with whom this work was carried out at John Innes Institute, Norwich, Great Britain. *R. leguminosarum*_biovar_viciae and *R. meliloti* lacking symbiotic (Sym) plasmids are these strains that have been cured of their Sym plasmids. They were grown in minimal medium[21] and total yeast (TY) medium when required[22].

Test for strain specificity for the production of *rhi* gene inducer.

Late exponential growth phase cultures of *R. leguminosarum*_biovar_viciae and *R. meliloti* with and without regulatory gene (*rhi*R) were tested for synthesis of the *rhi* gene inducer by alkaline phosphatase assay[22] using a *rhi* C::*pho*A fused gene.

Test for the effect of cell density on *rhi* gene expression.

Strain of *R.leguminosarum*_biovar_viciae carrying *rhi*A::*lac*Z fused gene with and without symbiotic plasmid were grown at 28^{0}C in liquid TY medium[22], shaken at 180 rev min^{-1} for 72 hours. Cell growth by viable plate count method and β-galactosidase activity[22] were determined during the incubation period at an interval of 6 hours.

Mutation experiment.

Transposon (Tn5) mutagenesis was carried out on mid-logarithmic phase cells of *R. leguminosarum*_biovar_viciae. One thousand one hundred and fifty mutants were screened on plate for the production of *rhi* gene inducer by alkaline phosphatase assay as described by[22] Miller. Nineteen mutants found defective in the synthesis of the inducer were tested for alkaline phosphatase activity by liquid assay[22]. Two controls (positive and negative) were prepared along with the mutants. The mutants were grouped into classes using respective 0-500, 501-1000, 1001-1500 and 1501- above Miller units of alkaline phosphatase.

Assay for β-galactosidase and alkaline phosphatase activities.

Methods described by[22] Miller were employed. That of b-galactosidase was slightly modified and carried out as follows. Reaction mixture contained 0.9 ml culture and 0.2 ml isopropyl-thio-b-D-galactoside. The reaction was stopped after five minutes at room temperature (27^{o}C) with 0.5 ml 1 M Na_2Co_3. Optical density of the mixture was read at 600nm and 420nm. The latter density was corrected to obtain the actual optical density. Units of b-galactosidase activity (Miller units) was defined as the absorbance at 420nm and 600nm per minute per millilitre of the reaction product under this assay condition.

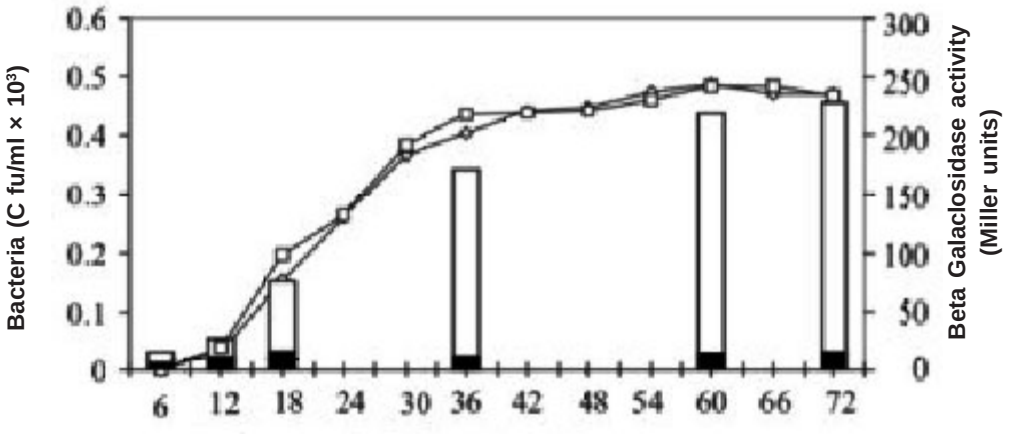
RESULTS

Phosphatase activity for synyhesis of *rhi*R inducer by *R. meliloti* was low relative to that of *R. leguminosarum* with and without symbiotic plasmid Table 1. Growth phase influenced *rhi* genes action Fig . 1. The colony forming units increased with incubation time in *R. leguminosarum* in the presence and absence of symbiotic plasmid. The two bacterial strains showed similar growth pattern without any significant difference. However, there was an increase in the activity of b-galactosidase with increased cell population in the presence of symbiotic plasmid with highest value of 237.5 Miller units in comparison with 12.5-17.5 Miller units for strain lacking symbiotic plasmid Fig. 1. This activity was highest in the exponential phase with pronounced increase in the mid-logarithmic phase than in any other growth phases.

Table 1: Strain specificity of *R. leguminosarum* bv. viciae for the production of *rhi* genes inducer.

Strain	Alkaline Phosphatase Activity (Miller units)
R. leguminosarum bv. Viciae:	
With Sym Plasmid	3000
WithoutSym Plasmid	900
R. meliloti:	
With Sym Plasmid	200
Without Sym Plasmid	50

Table 2 shows that the mutants were defective in the production of *rhi* genes inducer at various levels. Class I mutants (low-producers) having the inducer synthetic ability of 145-250 Miller units of phosphatase activity respectively comprised of mutant numbers 9, 15 and 16. Class II mutants (moderate-producers) were mutant numbers 1, 3, 4, 5, 6, 8, 11 and 14 with 625-896 Miller units. Class III mutants (high-producers, 1031-1375 Miller units) were made up of mutant numbers 2, 7, 10, 12, 13, 17 and 18 while class IV (super-producer) has a single member, number 19 showing formation ability of inducer at and 1563 Miller units. Relative to the negative and positive controls Table 2.



Legend: /Δ: Cell density (: without plasmid, Δ: with plasmid), / : Plasmid (: without plasmid, : with plasmid).

Fig. 1. Effect of cell density and symbiotic plasmid on *rhi* genes expression.

DISCUSSION

Synthesis of large amount of *rhi* genes inducer by the *R. leguminosarum* than *R. meliloti* indicates that production of this metabolite is specific for the former bacterium and thus suggests that only certain rhizobial species form the compound. The inability of *R. meliloti* to express the *rhi* genes at high level was possibly due to presence of other genes located on the symbiotic plasmid which could block the production of appropriate metabolite to induce the *rhi* genes expression.

β-galactosidase activity highest in the exponential phase with pronounced increase in the mid-logarithmic phase than in any other growth phases indicates that *rhi* genes function optimally when the cells are in the active stage of their growth. Thus, the regulation of *rhi* genes by RhiR is growth phase and cell density dependent. Stationary phase cells appeared less active than logarithmic phase cells. Although, diversity in production of and response to N-acyl homoserine lactones is possible[23]; the result obtained here is similar to that reported by Fuqua *et al.*,[10] and Gao *et al*.,[11]. Fuqua *et al.*,[10] said that LuxR-LuxI family of transcriptional regulators are cell density dependent in *Vibrio, Erwinia, Pseudomonas and Agrobacterium*. Gao *et al*.,[11] showed that rate of growth and symbiotic nodulation were directly related to the quorum-sensing system in *Mesorhizobium huakuii*. Many plant-associated bacteria regulate gene expression in a cell density-dependent manner by using quorum sensing via *N*-acyl homoserine lactones (AHLs). These AHLs pass out of and into bacterial cells. As the population of bacteria increases, so does the concentration of AHLs[24]**.**

Table 2: Effect of mutation on *rhi* genes inducer.

Strain	Alkaline Phosphatase Activity (Miller units)
Mutant 1	896.12
Mutant 2	1375.44
Mutant 3	781.5
Mutant 4	625.2
Mutant 5	812.76
Mutant 6	885.7
Mutant 7	1031.58
Mutant 8	875.28
Mutant 9	250.08
Mutant 10	1239.98
Mutant 11	896.12
Mutant 12	1146.2
Mutant 13	1250.4
Mutant 14	812.76
Mutant 15	145.88
Mutant 16	187.56
Mutant 17	1052.42
Mutant 18	1062.84
Mutant 19	1563
Negative control 1	187.56
Negative control 2	239.66
Positive control 1	1323.34
Positive control 2	1239.88

The data on mutation implies that Class I mutants were strongly defective in *rhi* genes induction, class II were averagely defective and class IV mutant *rhi* genes were enhanced by the mutation to express the genes better than the wild type. Class III appeared unaffected being related to the wild-type strains (positive controls 1 and 2). This could be because *rhi* genes in this class were not affected by the Tn5 mutation to lack or produce the metabolite at lower or higher level in contrast to the last two Classes I and II.

Conclusion

Synthesis of *rhi* genes inducer was specific for *R. leguminosarum* biovar viciae. The bacterium expressed *rhi* genes in a cell density dependent pattern. Symbiotic plasmid is vital for the action of *rhi* genes; *rhi* genes induction is under the influence of *rhi*R which is located on the symbiotic plasmid. Tn-5 mutation affected the *rhi* genes induction.

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