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The products of *Rhizobium* genes, *psi* and *pss*, which affect exopolysaccharide production, are associated with the bacterial cell surface

J. W. Latchford,[1] D. Borthakur[2] and A. W. B. Johnston[3]*

[1]*School of Ocean Sciences, University College of North Wales, Menai Bridge, Gwynedd LL59 0LY, UK.*
[2]*Biotechnology Program, University of Hawaii, 3050 Maile Way, Honolulu, Hawaii 96822, USA.*
[3]*School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK.*

Summary

Translational fusions between a mutant *phoA* (lacking its promoter, ribosomal binding site and signal peptide sequence) and *Rhizobium* 'symbiotic' genes were isolated. Since these fusions expressed alkaline phosphatase (AP), the product of *phoA*, the genes into which *phoA* was inserted apparently specify proteins located in the bacterial periplasm or cell membrane, the compartment in which AP has activity. These genes were *psiA* and genes upstream of *psiA* (*psiA* is required for normal nodule development and strains with multicopy *psiA* fail to make exopolysaccharide (EPS) and to nodulate). Fusions between *phoA* and *pss* (*exo*) genes, which are required for EPS production, also resulted in the expression of AP indicating that products of these *pss* genes were located at the cell surface. Using *gus* fusions to *psiA* and *pssA*, we found that the former was expressed in N_2-fixing bean root nodules but the latter was not.

Introduction

In the symbiont of *Phaseolus* beans, *Rhizobium leguminosarum* biovar *phaseoli* (*R.l.* bv. *phaseoli*), as in other strains of *Rhizobium* (see Long, 1989; Young and Johnston, 1989), many genes needed for nodulation, host-range specificity and the ability of the bacteria to fix N_2 are on large 'symbiotic' (Sym) plasmids (Hawkins and Johnston, 1988; Lamb *et al.*, 1985; Quinto *et al.*, 1982; Borthakur and Johnston, 1987; Borthakur *et al.*, 1987). The ability of strains of *Rhizobium* to make a high molecular-weight acidic exopolysaccharide (EPS), which accounts for the mucoid appearance of many species, is important for the bacteria to nodulate and/or to fix nitrogen. Different mutant strains defective in their ability to make this polymer have been shown, variously, to be unable to nodulate, to induce nodules that are devoid of bacteria and therefore fail to fix N_2 or, in some cases, to be unaffected in their ability to induce normal N_2-fixing nodules (reviewed by Gray and Rolfe, 1990). Despite the importance of the production of the EPS, it has been shown that at least in some species of rhizobia the Sym plasmid is not required for the production of this polymer.

The species of *Rhizobium leguminosarum* comprises three biovars, namely, *viciae* (which nodulates peas, vetches and lentils), bv. *trifolii* (nodulates clovers) and bv. *phaseoli* (whose hosts include *Phaseolus* beans); in this species, the host-range specificity is determined by genes on the Sym plasmid of each biovar. In *R. leguminosarum* strains that have lost their Sym plasmid, the quantity and quality of the EPS does not differ from that of wild-type strains (Borthakur *et al.*, 1988; J. A. Downie, W Dudman and V. M. Morris, personal communications). Nevertheless, in both *Rhizobium meliloti* (whose hosts include alfalfa) and *R.l.* bv. *phaseoli*, there are genes on the Sym plasmid which affect the synthesis of EPS. In the former, mutations in a gene, *syrM*, which is involved in regulating *nod* gene transcription, cause an enhanced level of production of the succino-glycan EPS for reasons that are not clear at present (Mulligan and Long, 1989). In *R.l.* bv. *phaseoli*, two regions on the Sym plasmid do affect the amount of EPS that is made and genetic alterations of these regions have major effects on symbiotic N_2 fixation ability (Borthakur and Johnston, 1987). Borthakur *et al.* (1985) identified a gene termed *psi* (polysaccharide inhibition) with the following properties. It is on Sym plasmid pRP2JI of a strain of *R.l.* bv. *phaseoli*, near *nod* and *nif* genes. When present in multicopy plasmids in *R.l.* bv. *phaseoli* or *R.l.* bv. *viciae* it prevents production of EPS and virtually abolishes nodulation ability of their respective hosts (Borthakur *et al.*, 1985). *psi* mutant strains induce nodules on *Phaseolus* beans which are completely defective in N_2 fixation ability (Borthakur *et al.*, 1985). Thus, *psi* is required for nodule development but its precise role in the process is unknown. *psi* was sequenced and was deduced to encode a protein of M_r 10 kDa with a hydrophobic *N*-terminus and a hydrophilic

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C-terminus, suggesting that the product of the sequenced *psi* gene is located in one of the bacterial membranes. It was found by deletion analysis that the promoter of *psi* lies at least 800 bp upstream of its translational start and that insertional mutations upstream of the sequenced *psi* gene abolish the inhibitory effects of cloned *psi*, presumably via a polar effect on *psi* (Borthakur and Johnston, 1987).

The inhibitory effects of multicopy *psi* on nodulation and EPS production were overcome when the bacteria also contained cloned copies of one of two other *Rhizobium* genes. One of these, which is also on the Sym plasmid, was termed *psr* (polysaccharide restoration). The introduction of cloned *psr* DNA into strains with multicopy *psi* restored the mucoid, EPS⁺ colony morphology and the ability of these strains to nodulate. This is because *psr* repressed transcription of *psi* (Borthakur and Johnston, 1987). Another gene, *pssA* (corresponding to ORF II in Borthakur *et al.*, 1988) and which is not on the Sym plasmid, when cloned in a plasmid vector and introduced into a strain with multicopy *psi* also overcame the inhibitory effects of multicopy *psi* on EPS production and nodulation ability. *pssA* is required for the synthesis of EPS; mutations in it (and in DNA upstream of it) abolish production of the polymer (Borthakur *et al.*, 1986). Sequence analysis revealed that the product of *pssA*, like Psi, has a hydrophobic *N*-terminus and it was suggested that PssA might also be membrane-associated and that there might be an interaction between PssA and Psi such that when both were overproduced, the relative concentrations of the two proteins would be close to normal and that this would account for the ability of multicopy *pssA* to overcome the inhibitory effects of multicopy *psi*.

Gray *et al.* (1990), working with a wide host-range strain (NGR234) of *Rhizobium*, reported a similar phenomenon to that which we had observed concerning the interaction between *psi* and *pssA*. They identified and cloned a gene, *exoY*, which was required for EPS synthesis, and found, by sequence analysis, that it was similar to *pssA*. Furthermore, *exoY* was shown to be functionally equivalent to a gene in the *exoF* locus of *R. meliloti* (Zhan *et al.*, 1990). Closely linked to *exoY* was another gene, termed *exoX*, which had similar properties to *psi* in that, when cloned in multiple copies, it inhibited EPS production; however, this effect could be overcome when *exoY*, like *pssA*, was also present in multiple copies. The deduced product of *exo* had some striking similarities to that of *psiA*: both are small polypeptides (M_r of approximately 10 kDa) and have very similar hydropnobicity profiles. However, apart from a short tract of 18 amino acids in the central regions of the proteins, *exoX* and *psiA* have little or no homology at the primary amino acid level (Gray *et al.*, 1990). Thus in *Rhizobium* strain NGR234, multiple copies of *exoY* can overcome the inhibitory effects of the cloned *exoX* gene, just as *pssA*, when cloned, can counteract the inhibition of EPS synthesis by the cloned *psi* gene. Similarly, Reed *et al.* (1991) found genes equivalent to *exoX* and *exoY* in *R. meliloti* and showed that they had the same behaviour relative to each other as the corresponding genes in strain NGR234. Note, though, that in *R.l.* bv. *phaseoli*, *psi* and *pssA* are unlinked, *psi* being on the Sym plasmid and *pssA* being on a separate replicon; in strain NGR234, however, and in *R. meliloti*, *exoX* and *exoY* are adjacent.

Here we show that the products of *psi* and *pssA* are associated with the bacterial membrane and that novel genes upstream of *psi* and *pssA* also encode proteins located in the bacterial membrane or periplasm. The evidence for this conclusion came from the use of an engineered transposon, Tn*phoA* (Manoil and Beckwith, 1985). Tn*phoA* is derived from Tn*5* and contains, near one end, a truncated version of the *Escherichia coli phoA* gene specifying alkaline phosphatase (AP) which lacks a promoter, ribosomal binding site and the 5' end of the gene encoding the signal peptide required for AP to be exported to the periplasm (the compartment in which AP has activity). Thus, insertions of Tn*phoA* only exhibit AP activity if the transposon inserts, in frame, into a transcribed gene that specifies a protein that is periplasmic in its location or which is associated with the membrane(s) such that the AP in the fusion protein is exposed to the periplasm. Tn*phoA* has been used in *R. meliloti* (Long *et al.*, 1988) to identify genes whose products are associated with the rhizobial membrane and which are involved in EPS synthesis in that species.

Studies of the interaction between the *psi* and *pss* system were also made.

Results

*Isolation of Tn*phoA *insertions in the* psi *region*

As judged by the deduced extremely hydrophobic nature of the *N* terminal half of the *psi* gene product, this region was proposed to insert into one of the surface membranes of *Rhizobium* (Borthakur and Johnston, 1987). In an attempt to confirm this, we isolated *psi–phoA* fusions and determined whether or not they expressed AP activity.

Recombinant plasmid pIJ1098 contains approximately 30 kb, including the sequenced *psi* gene, of Sym plasmid pRP2JI or *R.l.* bv. *phaseoli* strain 8002 (Lamb *et al.*, 1985). Following mutagenesis of pIJ1098 with Tn*phoA* (see the *Experimental procedures*), the pIJ1098::Tn*phoA* mutant plasmids were mobilized *en masse* into *Rhizobium* strain 8401 and transconjugant colonies were screened for any that were mucoid. Of 7000 such colonies, 98 were mucoid, indicating that they contained

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pIJ1098::*psi*Tn*phoA* mutant derivatives. Each of these mucoid colonies was replicated to medium containing X-P (5-bromo-4-chloro-3-indolyl phosphate, a chromogenic indicator for AP activity); two of them were bright blue, indicating that *phoA* was fused to a gene whose product is associated with the bacterial membranes and/or the periplasm.

These two mutant plasmids were isolated from the *Rhizobium* strains and were put into *E. coli* strain 803 by transformation and were then re-introduced into *Rhizobium* strain 8401; the *Rhizobium* transconjugants were EPS$^+$ and, on medium supplemented with XP, were blue. The locations of the Tn*phoA* insertions in the mutant plasmids were determined by restriction mapping and it was shown that one was in the previously identified *psi* gene, towards the 3' end of the gene, i.e. downstream of the 5' region which encodes the hydrophobic *N*-terminus of Psi. In this insertion, *phoA* was in the same orientation as *psi*. The other Tn*phoA* was in the same orientation, approximately 700 bp upstream of the translational start of *psi*. This insertion was within DNA in the same transcriptional unit as *psi* but which was not required for Psi function (Borthakur and Johnston, 1987). Presumably the Psi$^-$ phenotype caused by this insertion was due to a polarity effect of the transposon on transcription of *psi*. These observations indicated that the Psi is indeed associated with the bacterial envelope and that upstream of *psi* there is another gene, with no known function, which also encodes a polypeptide that is located in one of the membranes or the periplasm. This gene was termed *psiB* and the *psi* gene originally described and sequenced is now termed *psiA*.

Using *lacZ* fusions, it had been shown (Borthakur and Johnston, 1987) that transcription of *psiA* and the DNA up to 800 bp upstream of it is depressed by introduction of multicopy *psr*. Plasmid pIJ1426, which contains *psr* cloned in pKT230, was mobilized into the two derivatives of strain 8401 containing the *psi*::Tn*phoA* mutant derivatives of pIJ1098. Introduction of the cloned *psr* gene on pIJ1426 substantially reduced the activity of the *psi-phoA* fusions (Table 1). Thus, as expected, expression of these Tn*phoA* fusions is under the same type of control as the previously isolated *lac* fusions in the *psi* region.

Table 1. The effect of cloned *psr* on the activities of *psi*::Tn*phoA* fusions.

Rhizobium strains	Alkaline Phosphatase Activities of Strains Containing: no additional plasmid	pIJ1426
8401 pIJ1837 (*psiA*::Tn*phoA*)	340 ±14	48 ±3
8401 pIJ1838 (*psiB*::Tn*phoA*)	185 ±10	26 ±5

pssA *and genes upstream of* pssA *encode proteins associated with the cell surface*

Comparison of the sequence of *pssA* with *exoY* of *Rhizobium* strain NGR234, which is required for EPS synthesis in that species, shows that these two genes are related, having products with approximately 40% sequence identity (Gray *et al.*, 1990). Borthakur *et al.* (1988) showed that the *N*-terminus of PssA, deduced from the sequence of *pssA*, was hydrophobic, suggesting that PssA might be located in the bacterial membrane. In an attempt to confirm that *pssA* specifies a protein that is located in the rhizobial cell surface, a recombinant plasmid, pIJ1427, which contains 30 kb of *R.l.* bv. *phaseoli* DNA including *pssA*, was mutagenized with Tn*phoA* and the mutant derivatives were transferred to strain 8401 and plated on medium containing X-P. Of 2500 such derivatives, six gave bright blue colonies. Plasmid DNA was isolated from each of these strains and introduced into *E. coli* by transformation. It was found by restriction mapping that one of the insertions was in the vector, two were in *pssA* in the same orientation as that gene, and three insertions were in DNA upstream of *pssA*, the *phoA* again being in the same orientation as *pssA*. Each of the derivatives of pIJ1427 which expressed AP and which had insertions in the cloned DNA was transferred to a non-mucoid derivative of *R.l.* bv. *phaseoli* containing a *pssA*::Tn*5* mutation whose EPS$^-$ phenotype is corrected by pIJ1427; in all cases the transconjugants were non-mucoid. From this we deduced that these new Tn*phoA* insertions were in genes required for EPS production and that these were in the same transcriptional unit as *pssA*, i.e. the failure of the mutant plasmids with the insertions in the region upstream of *pssA* to correct the *pssA* mutant strain is presumably due to a polar effect on the transcription of *pssA*. The fact that these 'new' insertions were in *pss* genes was confirmed by introducing each of them by marker exchange into the genome of a wild-type *R.l.* bv. *phaseoli*; in all cases, the homogenotes were completely non-mucoid. With the fusions to *pssA*, the sites of the insertions of Tn*phoA* were downstream of the 5' region of *pssA* that encodes the hydrophobic part of PssA (Borthakur *et al.*, 1988). We deduce from these results that not only is the product of *pssA* associated with the membrane, but that *pssA* comprises part of a larger transcriptional unit which has other genes required for EPS production and whose products are also located at the cell surface.

Interaction between pss *and* psi *genes*

The presence of cloned *pss* genes overcomes the inhibitory effects on EPS synthesis and nodulation due to multicopy psi (Borthakur et al., 1988). This was not because pss inhibits transcription of psi (Borthakur et al., 1988). As shown here, the products of psiA, a gene

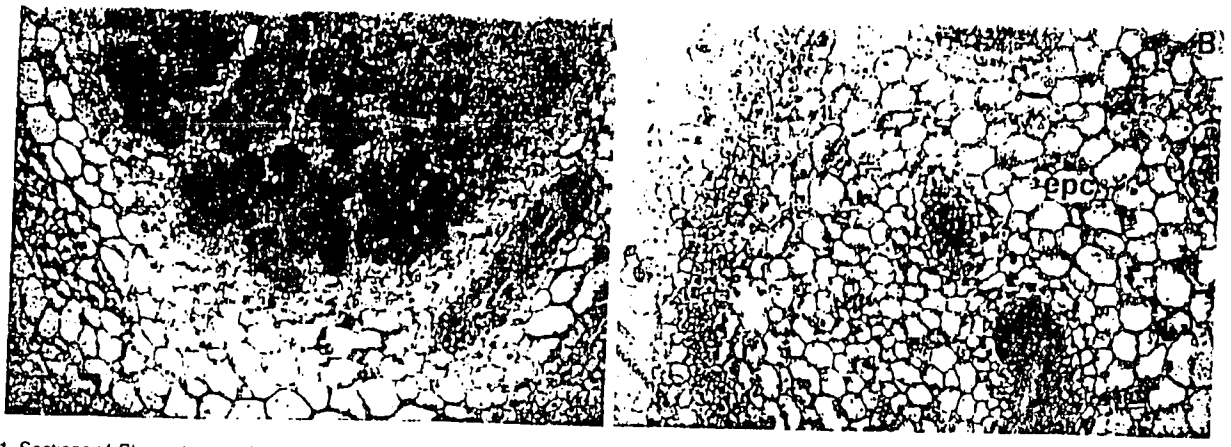

Fig. 1. Sections of *Phaseolus* nodules induced by wild-type (A) and *psiA* mutant (B) strains of *R.l.* bv. *phaseoli*. Note the absence of bacteria in the cells in the latter case. epc; empty plant cells. bcz; bacteroid-containing zone.

upstream of *psiA*, *pssA* and other *pss* genes upstream of *pssA* appear to be membrane-associated. It seemed possible, therefore, that there is some interaction between the products of PsiA (and/or the gene upstream of *psiA*) and Pss genes within the membrane and that the presence of multiple copies of *psiA* or *psiB* might affect the efficiencies with which the products of the *pss* genes are targeted to the cell surface (and/or vice versa).

To test this, we used the *pssA*–Tn*phoA* and *psi*::Tn*phoA* fusions described above. The logic was that if *pss* affected the targeting of PsiA or vice versa, this might be reflected in a drop in the activity of AP from the appropriate fusion because of reduced efficiency whereby AP part of the fusion was targeted to the periplasmic space or membrane.

The *psi*::Tn*phoA* insertions were each transferred by marker exchange from the appropriate pIJ1098 mutant plasmids into the Sym plasmid pRP2JI. Then a recombinant plasmid, pIJ1427, containing approximately 30 kb of cloned DNA including *pssA* and the additional *pss* genes identified here was introduced into these mutants. In addition, a mutant derivative of pIJ1427 (pIJ1471), containing a *pssA*–Tn*5* mutation was transferred to the two *psi*::Tn*phoA* mutant strains. AP activities in each of the transconjugant strains were assayed. In the strain with the *psiA*::*phoA* fusion, levels of AP activity were unaffected by the introduction of pIJ1427 or its *pssA* mutant derivative, pIJ1427, relative to the control containing no recombinant plasmid. In contrast, with the mutant strain containing the Tn*phoA* upstream of *psiA*, the level of AP was increased approximately fourfold by the presence of pIJ1427 (Table 2). This increase did not occur when pIJ1471 was present, showing that *pssA* was responsible for this increase in AP activity in this fusion. Since multicopy *pssA* does not affect the transcription of *psi* (Borthakur *et al.*, 1988), this observation indicates that multicopy *pssA* somehow increases the efficiency with which the product of the gene upstream of *psiA* is directed to the membrane(s) or periplasm, or in some other way enhances the level of exposure of AP to the periplasm.

To do the 'reciprocal' experiment, i.e. to test if multicopy *psi* affects expression of the *pss*–*phoA* fusions, each of the *pss*–*phoA* fusions described above was transferred by marker exchange from its position on pIJ1427 to the genome of *R.l.* bv. *phaseoli* strain 8002. Then into these mutants we introduced pIJ1393, which contains *psi* genes cloned in the vector pKT230. This plasmid had no effect on the level of AP activity from any of the *pssA*–Tn*phoA* fusions, indicating that *psi* genes had no effect on the efficiency with which these *pss* gene products are targeted to the bacterial cell surface.

Symbiotic effects of psi::*Tn*phoA *mutations*

Previously isolated *R.l.* bv. *phaseoli* strains with mutations in *psiA* or in DNA upstream of *psiA* induced aberrant, non-fixing nodules on *Phaseolus* beans (Borthakur *et al.*, 1985). The *psi*::Tn*phoA* mutations described here were transferred into pRP2JI by marker exchange and the resulting mutants were used to inoculate *Phaseolus* beans. Consistent with the earlier findings with Tn*5*-induced mutations in this region, these mutants induced small white nodules that failed to fix nitrogen, as determined by acetylene-reduction assays and the pale green colour of the leaves.

These Fix nodules were sectioned and examined by light microscopy; as shown in Fig. 1, they were devoid of bacteria, bacteroids and infection threads.

Table 2. Effect of cloned *pssA* on the activity of psi::Tn*phoA* fusions.

Strain	Alkaline Phosphatase Activities of Strains Containing: no additional plasmid	pIJ1427	pIJ1471
4292 *psiA*::Tn*phoA*	32 ±2.5	30 ±3	33 ±2.5
4292 *psiB*::Tn*phoA*	9 ±1.5	33 ±6	10 ±3

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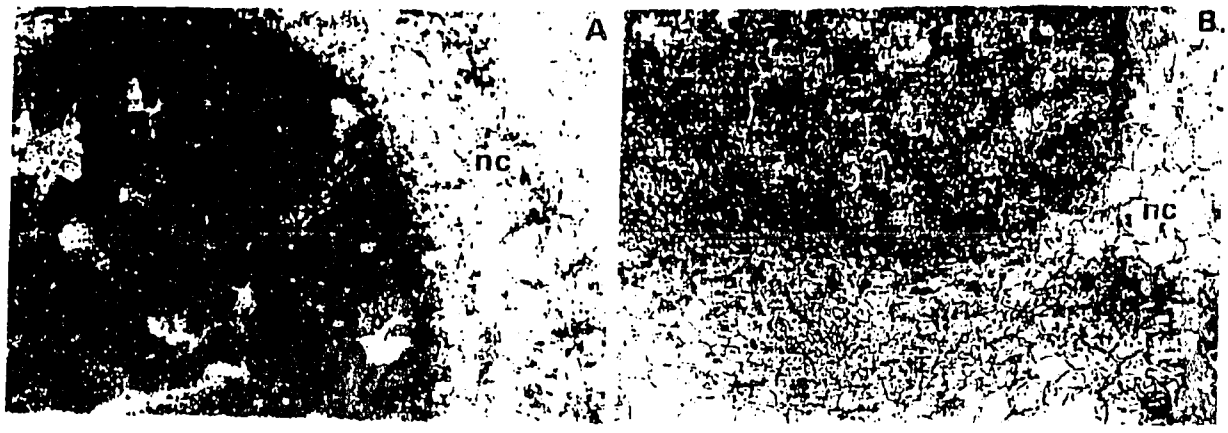

Fig. 2. Section of *Phaseolus* root nodules containing *R.l.* bv. *phaseoli gus* fusions to *pssA* (A) or *psiA* (B). The nodules were stained with X-Gluc as described in the text. Note the active staining with the *psi–uidA* fusion and the absence of staining with the *pss* fusion. nc, nodule cortex; bcz, bacteroid-containing zone. (See in colour on the cover of this issue.)

Expression of psi *and* pssA *in nodules*

Since mutations in the *psi* transcriptional unit abolished normal nodule development, it was apparent that the *psi* region must be transcribed during the infection process. To monitor expression of *psi* in the nodule, we made transcriptional fusions between *psi* and the *uidA* (*gus*) gene in essentially the same way in which the *psi*::Tn*phoA* mutations had been isolated. pIJ1098, the plasmid containing the *psi* genes, was mutagenized with Tn3*gus* (*Experimental procedures*) and, following transfer of the collection of mutant plasmids into strain 8401, 25 derivatives that had lost the ability to inhibit EPS synthesis were isolated, indicating that in these cases, the transposon had inserted into *psi* DNA. It was confirmed by restriction mapping that in these mutant forms of pIJ1098, Tn3*gusA* was in *psiA*, and that in three of these cases, it was in the same orientation as this gene. It was further found that these *psiA–gusA* fusion plasmids specified low-level (*c.* 50 units) glucuronidase activity in free-living cells and that the introduction of the cloned *psr* DNA caused a further fivefold reduction in the level of enzyme activity (not shown). On media containing the chromogenic indicator X-Gluc, the colonies containing these *psiA:gusA* fusions were very pale blue. The Tn3*gus* in *psiA* was introduced by marker exchange into the corresponding position in pRP2JI; the resulting mutant, as expected, induced non fixing 'empty' nodules on *Phaseolus* beans. Since this mutant induced nodules that were devoid of bacteria, it was obviously impossible to estimate the levels of glucuronidase in such strains within the nodule. Therefore, pIJ1098, which contains *psi* cloned in pLAFR1, was introduced into this strain in order to correct its Fix⁻ defect. The resulting transconjugant was Fix⁺ on *Phaseolus* beans and the nodules were examined histochemically for the presence of β glucuronidase. As shown in Fig. 2, it is clear that in the bacteroid containing zone of these nodules there is abundant enzyme activity, showing that *psiA* is expressed in these forms of the bacteria.

We also wished to determine whether *pssA* is expressed in the bacteroids. In essentially the same way as was described for the isolation of the *psiA*–Tn*gus* mutation, we mutagenized pIJ1427 (which contains *pssA*) with this transposon, and transferred the mutagenized plasmids *en masse* to a *pssA* mutant strain of *R.l.* bv. *phaseoli*. Non-mucoid transconjugants were purified and the mutant plasmids were introduced into *E. coli* by transformation; in one such mutant plasmid, pIJ1724, which was used for further study, *gus* was confirmed as being in the same orientation as *pssA*, and the derivative of strain 8401 containing pIJ1724 had constitutive, high-level (640 units) β-glucuronidase activity in free-living cells. The *pssA*::Tn3*gus* in pIJ1724 was introduced by marker exchange into the corresponding location in the genome of *R.l.* bv. *phaseoli* strain 4292. As expected, the mutant was EPS⁻ and, as described earlier, for other *pssA* mutant strains of this biovar (Borthakur *et al.*, 1986), induced N_2-fixing nodules on *Phaseolus* beans. These nodules were sectioned and examined for β-glucuronidase activity. As shown in Fig. 2, the bacteroids in such a nodule did not stain following exposure of the nodules to the chromogenic indicator, X-Gluc.

Discussion

The data presented here provide new information about the *psi* and *pss* (*exo*) genes of *R.l.* bv. *phaseoli*. Using transposon Tn*phoA*, it was shown that the product of the previously identified *psiA* gene is associated with the bacterial membrane or periplasm. Taken together with the extremely hydrophobic *N*-terminus of *psiA*, we favour the view that PsiA is, in fact, anchored in one of the membranes, consistent with its possession of an extremely

hydrophobic *N*-terminus (Borthakur *et al.*, 1987). We did not establish, though, if the protein was located in the inner or outer membrane of the bacteria. Furthermore, it was clear that there is another, novel gene upstream of *psiA* which also specifies a protein that is either in one of the membranes or in the periplasm, since a translational fusion of Tn*phoA* into this gene expressed AP activity. The function of this gene remains unknown; mutations in it abolish the effect of multicopy *psiA* on EPS production and prevent nitrogen fixation but this could be due simply to a polar effect of such insertion mutations on the transcription on *psiA* itself. It is known that the inhibitory effect of multicopy *psiA* on EPS synthesis can be overcome by the introduction of the cloned *pssA* gene, but this is not due to a reduction in the transcription of *psi* caused by the extra copies of *pssA* (Borthakur *et al.*, 1988). Also, as shown here, the presence of the cloned *pssA* did not affect the activity of AP determined by *psiA–phoA* fusions, indicating that the efficiency with which the PsiA is directed into the membrane(s) or periplasm is not altered by overproduction of the *pssA* gene product. The finding of active fusions between *pssA* and *phoA* helped to confirm the membrane location of PssA (something which had been predicted from sequence analysis; Borthakur *et al.*, 1988). The *exoY* gene of *R. meliloti* is similar in sequence to *pssA* and to *exoY* of strain NGR234; (Gray *et al.* 1990; Reed *et al.*, 1991). It was shown, also using *phoA* fusions to *exoY*, that the product of this gene, too, was likely to be associated with the cell surface (Long *et al.*, 1988). Some novel genes, involved in EPS production, which lay upstream of *pssA* and which are probably in the same transcriptional unit as that gene were also shown to express AP when *phoA* was fused to them. Long *et al.* (1988) also found that more than one gene involved in EPS synthesis in the *exoF* region of *R. meliloti* was associated with the cell surface, as deduced from the use of the same Tn*phoA* transposon. Reuber *et al.* (1991) reported that active insertions were also found in fusions to the *exoP*, *exoQ*, and *exoA* gene products.

Using these *pss–phoA* fusions in *R.l.* bv. *phaseoli* it was found that *psi* in multiple copy did not affect the activity of *phoA* fusions in *pssA* or of other fusions between *phoA* and the other, newly described, *pss* genes. It is still not clear why multicopy *pssA* overcomes the inhibitory effects of multicopy *psiA*. It was striking though, that the presence of multicopy *pssA* actually enhanced expression of AP from the fusion between *phoA* and the gene upstream of *psiA*; this was not due to an increase in the rates of transcription (Borthakur *et al.*, 1988) so must reflect an increase in the efficiency with which the product of that gene is targeted to the membrane or with which the AP part of the fusion protein is exposed to the periplasm; however, the precise mechanism behind this phenomenon is unknown.

Table 3. Strains and plasmids used in this study.

Strain/Plasmid	Relevant characteristics	Source/Reference
E. coli strain		
803	Used for routine transformation	Wood (1966)
C2110	*polA nal*^{R} – used as a source for Tn*phoA* mutagenesis	Stachel *et al.* (1985)
Rhizobium strain		
8002	Wild-type *R.l.* bv. *phaseoli*	Lamb *et al.* (1982)
8401	*R. leguminosarum* 8002 cured of Sym plasmid, also Str^R	Lamb *et al.* (1982)
Plasmid		
pRP2JI	Wild-type Sym plasmid of strain 8002	Lamb *et al.* (1982)
pIJ1098	Contains 30 kb of pRP2JI DNA cloned in pLAFRI and possesses cloned *psi* DNA	Lamb *et al.* (1985)
pIJ1393	Contains *psi* DNA cloned in Q-group plasmid pKT230	Borthakur *et al.* (1985)
pIJ1837	pIJ1098: *psiA*::Tn*phoA*	This work
pIJ1838	pIJ1098: *psiB*::Tn*phoA*	This work
pIJ1426	*psr* DNA cloned in pKT230	Borthakur and Johnston (1987)
pIJ1427	Contains 30 kb, including *pss* genes of *R.l.* bv. *phaseoli* cloned in pLAFR1	Borthakur *et al.* (1988)
pIJ1471	pIJ1427: *pssA*::Tn5	Borthakur *et al.* (1988)
pIJ1724	pIJ1427: *pssA*::Tn*gus*	This work

The work presented here confirms the importance of the *psi* and *pss* genes for symbiotic nitrogen fixation. *psi* mutant strains are not detectably altered in their ability to make EPS and, to date, such mutant strains have no obvious phenotype in free-living bacteria. However, the nodules that are induced by such strains completely fail to fix nitrogen and they are very aberrant relative to *Phaseolus* nodules induced by wild-type strains. No bacteria were seen within these Fix$^-$ nodules and there were no infection threads. Thus, these nodules resembled those that are induced by strains of *R. meliloti* which are deficient in the production of EPS or of the cyclic β,1-2 glucan normally made by these bacteria (reviewed by Gray *et al.*, 1991). Thus *psi* mutant strains represent another example in which the *Rhizobium* cells outside the root can induce the formation of nodule-like structures without actually entering the plant. It is not clear why such mutant strains should have lost the ability to induce normal nodules; clearly, *psi* can affect EPS production, admittedly under the artificial situation in which *psi* is present in multiple copies. If multiple copies of *psi* cause a reduction in EPS synthesis, it may be that a mutation in this gene causes an overproduction of the polymer. Although such a phenomenon is not observed in free-living bacteria, it is conceivable that this might occur during the infection process and that this is detrimental for normal nodule development. It is certainly the case that *psi* is expressed by bacteroids within the nodule, as shown here by direct

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cytochemical staining of the bacteroids containing *psiA–gusA* fusions. Note, though, that *psi* mutant strains abort the infection process prior to bacteroid formation, so its expression earlier in the infection process must be important for normal nodule development.

It had been shown that bacteroids of *Bradyrhizobium japonicum*, the symbiont of soybeans, make very little EPS in the nodules (Tully and Terry, 1985). Consistent with this was the finding that expression of *pssA–gusA* fusions was undetectable in bacteroids of *R.l.* bv. *phaseoli*, whereas this fusion is actively transcribed in free-living cells. Similarly, Reuber and Walker (1991) found that in alfalfa nodules *exoF* was not expressed in bacteroids but that there was some expression in the younger infection zone. This did not appear to be the case for *pssA*. This indicates that there may be some negative control which represses the transcription of the *pss* gene(s) in the bacteroid state. In *R. meliloti*, two regulatory genes, *exoR* and *exoS*, which serve to repress transcription of *exo* genes have been identified (Doherty *et al.*, 1988) but it remains to be seen if and how these genes are involved in the control of EPS production in nodules.

Experimental procedures

Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are shown in Table 3.

Microbiological techniques

Growth media and culture conditions for *Rhizobium* and *E. coli* were as in Beringer (1974). Plasmids were transferred by conjugation (see Figurski and Helinksi, 1979).

To mutagenize pIJ1098 (which contains *psi*) or pIJ1427 (which contains *pss*) with Tn*phoA*, the following procedure was used. Plasmid pRK609 (a kind gift from Graham Walker; Long *et al.*, 1988), which is a derivative of pRK2073 containing Tn*phoA*, was transferred by conjugation into the *E. coli polA* mutant strain C2110 selecting kanamycin resistance (Kan^R) (specified by Tn*phoA*). Since pRK609 cannot replicate in *polA* mutants, such Kan^R derivatives arise at low frequency, these being due to transposition of Tn*phoA* from pRK609 into the genome of C2110.

One Tn*phoA*-containing derivative of C2110 (C2110/Tn*phoA*) was purified. Plasmids pIJ1098 and pIJ1427 were introduced into C2110/Tn*phoA* by conjugation and the resulting strains were used as donors in triparental crosses (Figurski and Helinksi, 1979) in which *R. leguminosarum* strain 8401 was the recipient. By plating the products of the matings on medium containing Kan (specified by Tn*phoA*) plus tetracycline (Tet^R (specified by pLAFR1, the vector for the recombinant plasmids) plus streptomycin (to which strain 8401 is resistant), colonies of *R. leguminosarum* containing derivatives of each of the plasmids with random insertions of Tn*phoA* were obtained.

Similarly, to isolate derivatives of pIJ1098 and pIJ1427 with Tn*uidA* insertions into *psi* and *pssA*, respectively, these two plasmids were introduced by transformation into an *E. coli* strain with a chromosomally located insertion of Tn*uidA* (kindly supplied by B. Staskiewicz) and the same selection into strain 8401 as used for Tn*phoA* was employed except that selection for the joint transfer of Tet^R and ampicillin resistance (Amp^R) (specified by Tn*uidA* which is a derivative of Tn*3*) was made. Active insertions were identified by plating on media supplemented with X-P or X-Gus for Tn*phoA* and Tn*uidA*, respectively (these are the 'X-gal analogues' for alkaline phosphatase and β-glucuronidase activities, respectively). There was no background signal (i.e. colonies of unmodified *Rhizobium* controls were white on media containing these indicators, whereas strains containing active *phoA* fusions were bright blue on the appropriately supplemented media).

The transposons were transferred from their locations on recombinant plasmids to their corresponding locations in the *R.l.* bv. *phaseoli* genome by marker exchange (Ruvkun and Ausubel, 1981) using the P1-group plasmid, pPH1JI (Beringer *et al.*, 1978), to eliminate the recombinant plasmid. Alkaline phosphatase and β-glucuronidase activities were determined quantitatively as described by Miller (1972).

Plant work

Phaseolus beans (cv. Provider) were inoculated, grown, and assayed for N_2 fixation by the acetylene-reduction technique as described by Borthakur *et al.* (1985).

Examination of nodule structure using light microscopy

Nodules were fixed in glutaraldehyde, post-fixed in osmic acid, and embedded in LR white resin as described by Vandenbosch (1986). Sections (0.5 nm) were cut on a Reichert OMU3 microtome, and examined under a Zeiss Universal microscope.

In situ *estimation of* gus *activity (Jefferson* et al., *1987)*

Nodules were fixed in 0.3% formaldehyde in 10 mM MES, pH 5.6, 0.3 M mannitol for 45 min followed by several washes in 50 mM NaH_2PO_4 pH 7. All fixatives and substrates were introduced into tissues with brief vacuum infiltration. Sections (50 mm) were cut and histochemically stained with X-gluc (Research Organica Ltd; 1 mM in 50 mM NaH_2PO_4 pH 7 at 37°C). After staining, tissues were rinsed in 70% ethanol for 5 min, examined using Zeiss Universal microscope, and photographed using Kodak Ektochrom 64.

DNA manipulation

Restriction enzymes, and DNA ligase were used according to the manufacturers' instructions. Techniques involved in transformation of *E. coli* and restriction mapping were essentially as described by Maniatis *et al.* (1982).

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References

Beringer, J.E. (1974) R factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol* **84:** 188–198.

Beringer, J.E., Beynon, J.L., Buchanan-Wollaston, A.V., and Johnston, A.W.B. (1978) Transfer of the drug resistance transposon Tn*5* to *Rhizobium*. *Nature* **276:** 633–634.

Borthakur, D., Downie, J.A., Johnston, A.W.B., and Lamb, J.W. (1985) *psi*, a plasmid-linked *Rhizobium phaseoli* gene which inhibits exopolysaccharide production and which is required for symbiotic nitrogen fixation. *Mol Gen Genet* **200:** 278–282.

Borthakur, D., Barber, C.E., Lamb, J.W., Daniels, M.J., Downie, J.A., and Johnston, A.W.B. (1986) A mutation that blocks exopolysaccharide synthesis prevents nodulation of peas by *Rhizobium leguminosarum* but not of beans by *Rhizobium phaseoli* and is corrected by cloned DNA from the phytopathogen *Xanthomonas*.

Borthakur, D., Barker, R.F., Latchford, J.W., Rossen, L., and Johnston, A.W.B. (1988) Analysis of *pss* genes of *Rhizobium leguminosarum* required for exopolysaccharide synthesis and nodulation of beans: their primary structure and their interaction with *psi* and other nodulation genes. *Mol Gen Genet* **213:** 155–162.

Borthakur, D., and Johnston, A.W.B. (1987) Sequence of *psi*, a gene on the symbiotic plasmid of *Rhizobium phaseoli* which inhibits exopolysaccharide synthesis and nodulation and demonstration that its transcription is inhibited by *psr*, another gene on the symbiotic plasmid. *Mol Gen Genet* **207:** 149–154.

Borthakur, D., Lamb, J.W., and Johnston, A.W.B. (1987) Identification of two classes of *Rhizobium phaseoli* genes required for melanin synthesis, one of which is required for nitrogen fixation and which regulates transcription of the other. *Mol Gen Genet* **207:** 149–154.

Doherty, D., Leigh, J.A., Glazebrook, J., and Walker, G.C. (1988) *R. meliloti* mutants that overproduct the *R. meliloti* acidic calcofluor binding exopolysaccharide. *J Bacteriol* **170:** 1249–4256.

Figurski, D.H., and Helinksi, D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci USA* **76:** 1648–1652.

Gray, J.X., and Rolfe, B.G. (1990) Exopolysaccharide production in *Rhizobium* and its role in invasion. *Mol Microbiol* **4:** 1425–1429.

Gray, J.X., Djordjevic, M.A., and Rolfe, B.G. (1990) Two genes that regulate exopolysaccharide production in *Rhizobium* sp. NGR234: DNA sequences and resultant phenotypes. *J Bacteriol* **172:** 193–203.

Gray, J.X., de Mangd, R.A., Rolfe, B.G., Johnston, A.W.B., and Luglenberg, B.J.J. (1991) The role of the *Rhizobium* cell surface during symbiosis. In *Plant Microbe Interactions*. Verma, D.P.S. (ed.). *CRC Press*, in press.

Hawkins, F.K.L., and Johnston, A.W.B. (1988) Transcription of a *Rhizobium leguminosarum* biovar *phaseoli* gene needed for melanin synthesis is activated by *mlA* of *Rhizobium* and *Klebsiella pneumoniae*. *Mol Microbiol* **2:** 331–337.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile fusion marker in higher plants. *EMBO J* **6:** 3901–3907.

Lamb, J.W., Hombrecher, G., and Johnston, A.W.B. (1982) Plasmid determined nodulation and nitrogen fixation abilities in *Rhizobium phaseoli*. *Mol Gen Genet* **186:** 449–452.

Lamb, J.W., Downie, J.A., and Johnston, A.W.B. (1985) Cloning of the nodulation (*nod*) genes of *Rhizobium phaseoli* and their homology to *R. leguminosarum nod* genes. *Gene* **4:** 235–241.

Long, S.R. (1989) Legume nodulation: life together in the underground. *Cell* **56:** 203–214.

Long, S.R., McCune, S., and Walker, G.C. (1988) Symbiotic loci of *Rhizobium meliloti* identified by random Tn*phoA* mutagenesis. *J Bacteriol* **170:** 4257–4265.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Manoil, C., and Beckwith, J. (1985) Tn*phoA* transposon probe for protein export signals. *Proc Natl Acad Sci USA* **82:** 8129–8133.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor New York: Cold Spring Harbor Laboratory Press.

Mulligan, J.T., and Long, S.R. (1989) A family of activator genes regulates expresssion of *R. meliloti* nodulation genes. *Genetics* **122:** 7–18.

Quinto, C., de la Bega, H., Flores, M., Fernandez, L., Ballods, T., Soberon, G., and Palacios, R. (1982) Reiteration of nitrogen fixation gene-sequences in *Rhizobium phaseoli*. *Nature* **299:** 724–726.

Reed, J.W., Capage, M., and Walker, G.C. (1991) *Rhizobium meliloti exoG* and *exoJ* affect the ExoX/ExoY system for nodulation of exopolysaccharide production. *J Bacteriol* **173:** 3776–3788.

Reuber, T.L., and Walker, G.C. (1991) Regulation of *Rhizobium meliloti exo* genes in free-living cells and *in planta* analysed using Tn*phoA* fusions, in press.

Reuber, T.L., Reed, J.W., Glazebrook, J., Urzainqui, A., and Walker, G.W. (1991) Analyses of the roles of *R. meliloti* exopolysaccharides in nodulation. In *Advances in Molecular Genetics of Plant–Microbe Interactions*. Vol. 1. Hennecke, H., and Verma, D.P.S. (eds). Dordrecht: Klaiver Academic Publishers, pp. 182–188.

Ruvkun, G.B., and Ausubel, F.M. (1981) A general method for site directed mutagenesis in prokaryotes. *Nature* **289:** 85–88.

Stachel, S.E., An, G., Flores, G., and Nester, E.W. (1985) A Tn*3*-*lacZ* transposon for the random generation of β-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. *EMBO J* **4:** 891–898.

Tully, R.E., and Terry, M.E. (1985) Decreased exopolysaccharide synthesis by anaerobic and symbiotic cells of *Bradyrhizobium japonicum*. *Plant Physiol* **79:** 445–450.

Vandenbosch, K.A. (1986) Light and electron microscopic visualisation of uricase by immunogold labelling of sections of resin embedded soyabean nodules. *J Microscopy* **143:** 187–197.

Wood, W.B. (1966) Host specificity of DNA produced by *E. coli* mutations affecting the restriction and modification of DNA. *J Mol Biol* **16:** 118–133.

Young, J.P.W., and Johnston, A.W.B. (1989) The evolution of specificity in the *Rhizobium*-legume symbiosis. *Trends Ecol Evol* **4:** 341–349.

Zhan, H., Gray, J.X., Levery, S.B., Rolfe, B.G., and Leight, J.A. (1990) Functional and evolutionary relatedness of genes for exopolysaccharide synthesis in *Rhizobium meliloti* and *Rhizobium* sp. strain NGR234. *J Bacteriol* **172:** 5245–5250.

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