The *psi* operon of *Rhizobium leguminosarum* biovar *phaseoli*: identification of two genes whose products are located at the bacterial cell surface

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We have delineated three short open reading frames, *psiA*, ORF-P and *psiB* within the *psi* operon of *Rhizobium leguminosarum* biovar *phaseoli*. *psiA*, in a multi-copy plasmid, causes inhibition of exopolysaccharide synthesis in *R. leguminosarum*. In addition, the suppression of exopolysaccharide synthesis due to the multi-copy *psiA* caused *R. leguminosarum* strains to stain with the dye calcofluor, a response that does not occur with wild-type strains of this species. Insertions of a defective *phoA* gene (lacking its promoter, ribosomal binding site and leader sequence) into *psiA* and *psiB* were isolated and the precise locations of the insertions were established. PsiA-PhoA and PsiB-PhoA protein fusions were found to express alkaline phosphatase activity indicating that PsiA and PsiB span the inner membrane or are translocated across it.

Keywords: Calcofluor, exopolysaccharide, *phoA*-fusions, *Rhizobium*

INTRODUCTION

Strains of the bacterial genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* induce nitrogen-fixing nodules on legumes. The particular species of legume that is nodulated by a given species or strain of *Rhizobium* is determined by a suite of nodulation (*nod*) genes which has, in several cases, been located on large 'symbiotic' plasmids, close to other genes required for symbiotic nitrogen fixation (Downie & Johnston, 1988).

Many of these *nod* genes are involved in the synthesis of signal lipooligosaccharides that induce early steps in nodulation (see Fisher & Long, 1992). In addition to these *nod* genes, other *Rhizobium* genes are necessary for successful nodulation. Notably, it has been shown that the ability to produce the high molecular mass acidic exopolysaccharide (EPS) is required for the induction of nitrogen-fixing nodules. For example, *R. meliloti* mutants

which fail to make the succinoglycan EPS (*exo*) induce nodules on alfalfa, but the nodules are devoid of bacteria and consequently fail to fix nitrogen (Leigh *et al.*, 1985). In *R. leguminosarum* bv. *viciae*, EPS deficient mutants fail to induce nodules on peas (Borthakur *et al.*, 1986), but such mutants can induce nitrogen-fixing nodules on determinate nodulating plants such as *Phaseolus* beans (Borthakur *et al.*, 1986; Hotter & Scott, 1991).

In *R. meliloti*, where the most detailed genetic analyses have been conducted (see Leigh & Coplin, 1992; Gray & Rolfe, 1990), most of the *exo* genes are located in a cluster on a large indigenous plasmid, different from the 'pSym' that contains the *nod* and *nif* genes (Long *et al.*, 1988; Finan *et al.*, 1986; Hynes *et al.*, 1986). However, a gene was found located close to several *nod* and *nif* genes which could have marked effects on EPS synthesis in a strain of *R. leguminosarum* bv. *phaseoli*, (Borthakur *et al.*, 1985; Lamb *et al.*, 1985; Davis & Johnston, 1990a,b; Hawkins & Johnston, 1988). This gene was termed *psiA* (polysaccharide inhibition) and was detected initially because *Rhizobium* strains containing the cloned *psi* gene were non-mucoid. These EPS⁻ strains were defective in nodulation of peas and *Phaseolus* beans by strains of *R. leguminosarum* biovars *viciae* and *phaseoli*, respectively (Borthakur *et al.*, 1985), and a strain carrying a *psiA::Tn5* mutation induced on *Phaseolus* beans non-fixing nodules

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Abbreviations: AP, alkaline phosphatase; EPS, exopolysaccharide; NTG, nitrosoguanidine; X-P, 4-chloro-5-bromo-3-indolyl phosphate.

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which contained few, if any, bacteria (Borthakur *et al.*, 1985; Latchford *et al.*, 1991).

psiA is predicted to encode a small (M_r 10000) protein with a strongly hydrophobic NH_2 -half and a hydrophilic carboxy-domain, suggesting that it may be anchored in the cytoplasmic membrane of the bacteria (Borthakur & Johnston, 1987). Further, some translational fusions between *psiA* and a defective *phoA* gene that does not encode a signal peptide sequence were found to express alkaline phosphatase (AP) activity, providing evidence that *psiA* encodes a protein which transverse the bacterial inner membrane (Latchford *et al.*, 1991).

Bacteroids make little or no EPS (Tulley & Terry, 1985) and it was proposed (Borthakur *et al.*, 1985) that a normal function of *psiA* was to shut down EPS synthesis by bacteroids; indeed, *psiA* is expressed at high levels in bacteroids but, normally, only weakly in free-living *R. leguminosarum* bv. *phaseoli* (Latchford *et al.*, 1991). It was suggested that increased levels of Psi protein resulting from the cloned *psiA* could inhibit EPS synthesis in free-living *Rhizobium*.

Genetic evidence indicated that *psiA* was part of a longer transcriptional unit. Recombinant plasmids containing *psiA*, plus as much as 1 kb upstream of the gene, only conferred a Psi^+ phenotype if the cloned DNA was transcribed from a constitutive promoter in the cloning vector. However, if the cloned DNA extended to 1.3 kb upstream of *psiA*, the inhibitory effects of cloning this region were independent of the orientation. There is good evidence that this 1 kb region contains at least one other gene since Latchford *et al.* (1991) isolated translationally active *phoA* insertions within it.

Transcription of the *psi* region is repressed by the gene, *psrA*, which is about 13 kb from *psiA* on the symbiotic plasmid pRP2JI (Borthakur & Johnston, 1987). The predicted sequence of *PsrA* revealed an α helix-turn- α helix motif, which has striking similarity to the DNA-binding domains of several other prokaryotic transcriptional regulators (Mimmack *et al.*, 1994). Introducing a plasmid carrying *psrA* restored EPS production to a strain containing multi-copy *psiA* (Borthakur & Johnston, 1987).

By a very different mechanism, the cloning of another gene, termed *pssA*, also suppressed the EPS^- phenotype conferred by multi-copy *psiA* (Borthakur *et al.*, 1988). *pssA* is required for EPS synthesis (Borthakur *et al.*, 1986) and, like *psiA*, specifies a membrane-associated protein. *psiA* does not affect the transcription of *pssA* and *pssA* does not affect the transcription of *psiA* (Borthakur *et al.*, 1988). It was suggested that there is an association between the PsiA and PssA proteins in the membrane and an excess of PsiA relative to PssA causes the failure to make EPS; but, when *pssA* itself is also cloned, the correct 'balance' between PsiA and PssA is restored, allowing EPS synthesis (Borthakur *et al.*, 1986).

A gene (termed *exoX*) whose behaviour is similar to that of *psiA* was later identified in *R. meliloti* and *Rhizobium* sp. NGR234 (Reed *et al.*, 1991; Gray *et al.*, 1990). As with

psiA, cloning of *exoX* causes a dominant EPS^- phenotype in rhizobia. There are structural similarities in the ExoX and PsiA proteins; they have very similar sizes and almost superimposable hydropathy profiles, but amino acid sequence identity between ExoX and PsiA is restricted to a short (14 amino acid) tract close to the junction of the hydrophobic and hydrophilic portions of the proteins (see Gray *et al.*, 1990). The ability of *exoX* to inhibit EPS synthesis can be overcome by the presence of multiple copies of another gene, *exoY* (Reed *et al.*, 1991; Gray *et al.*, 1990) which has a significant sequence identity to *pssA* (see above). The model put forward (Gray & Rolfe, 1990) for the way in which ExoY overcomes multi-copy *exoX* was very similar to that suggested by Borthakur *et al.* (1986) for the interaction between PsiA and PssA.

In this paper we present the sequence of the *psi* region, describe the structure of *psiB*, and identify the precise locations of the 'active' *psi-phoA* fusions. Further, we describe a surprising phenomenon which relates the presence of *psiA* to the ability of *Rhizobium* colonies to stain with the optical brightener calcofluor.

Table 1. Bacterial strains and plasmids

Bacteria	Characteristics	Source
<i>E. coli</i>		
803	Used for routine transformations	Wood (1966)
JM101	Used as host for pUC sequencing vectors	Sambrook <i>et al.</i> (1989)
<i>R. leguminosarum</i>		
8401	Cured of pSym pRP2JI; also Str^r	Lamb <i>et al.</i> (1982)
J95	Exo mutant of 8401	This work
Plasmids		
pRP2JI	pSym of <i>R. leguminosarum</i> bv. <i>phaseoli</i>	Lamb <i>et al.</i> (1982)
pBIO139	<i>R. leguminosarum pssA</i> gene cloned in pKT230	This work
pIJ1393	<i>psiA</i> and <i>psiB</i> cloned in pKT230	Borthakur <i>et al.</i> (1985)
pIJ1614	<i>psiA</i> cloned in pKT230	Borthakur (1986)
pIJ1098	30 kb of pRP2JI containing <i>psi</i> and <i>nod</i> genes in pLAFR1	Lamb <i>et al.</i> (1985)
pBIO110 pBIO112 pBIO113 pBIO114 pBIO115 pBIO118 pBIO124 pBIO125 pBIO127 pBIO129 pBIO130	Derivatives of pIJ1098 containing <i>TnphoA</i> in <i>psi</i>	Latchford <i>et al.</i> (1991) and this work
	Fragments of the <i>psi</i> region cloned in pUC18 (see Fig. 2)	This work

METHODS

Bacterial strains and plasmids. These are shown in Table 1.

Media and general growth conditions. These were as described by Beringer (1974). 4-Chloro-5-bromo-3-indolyl phosphate sodium salt (X-P) was incorporated into solid media at $40 \mu\text{g ml}^{-1}$ and, where appropriate, 0.02% (w/v) calcofluor (Sigma). Triparental 'patch cross' matings were done as described by Buchanan-Wollaston *et al.* (1980) using pRK2013 as the 'helper' plasmid.

Isolation of EPS⁻ mutants of *R. leguminosarum*. Approximately 10^8 cells of *R. leguminosarum* were spread on a plate containing TY medium. After 4 h at 28 °C, a few crystals of nitrosoguanidine (NTG) were placed in the centre of the plate. After 3 d at 28 °C there was a zone of clearing around the NTG, from the edge of which cells were scraped off with a sterile loop, resuspended in 20% glycerol and plated out at appropriate dilutions on TY medium. Non-mucoid colonies were identified after 3 d of growth at 28 °C.

DNA manipulations and sequencing. Routine DNA manipulations (restriction digestions, ligation transformation, etc.) were done essentially according to Sambrook *et al.* (1989). DNA sequencing of the 1700 bp *psi* region was done by compiling a restriction map of this fragment, aided, in part, by the knowledge of some sequences upstream of *psiA* (Borthakur, 1986). Appropriate fragments were cloned into pUC18 vectors and double-stranded DNA sequencing was done using the Sanger dideoxy method. Sequence data were obtained and compiled using an automated ALF (Pharmacia) sequencer according to the manufacturer's instructions. To sequence the junctions between *phoA* and the *psi* genes, an oligonucleotide, KH107, corresponding to the 5' end of the *phoA* gene was synthesized, purified by standard methods and used as a primer in sequencing reactions.

RESULTS

Multicopy *psi* causes *R. leguminosarum* to stain with the optical brightener calcofluor

Much of the work on *exo* genetics in *R. meliloti* has hinged upon the observation that the EPS of this species stains with the optical brightener calcofluor when the colonies are viewed under UV light; mutations in different *exo* loci variously abolish or reduce this staining (see Leigh & Coplin, 1992). In fact, colonies of several strains of *R. meliloti* do not have a mucoid appearance, and *exo* mutants were isolated by their failure to fluoresce. For reasons which are not clear, colonies of *R. leguminosarum* do not fluoresce with calcofluor under UV, despite their pronounced mucoid appearance and we have failed to observe staining with the dye with several wild-type strains and biovars of this species both on complete (TY) and minimal (Y) media (unpublished observations).

During the course of this survey, we examined the calcofluor staining of *R. leguminosarum* strains and mutants and came across what appeared to be a paradoxical result. A derivative of *R. leguminosarum* harbouring the plasmid pIJ1393 (which contains *psiA* plus upstream sequences) fluoresced when examined under UV light (Fig. 1) after growth on TY medium supplemented with calcofluor. This was due to the *psiA* gene since *R. leguminosarum* strain 8401 carrying another plasmid, pIJ1614, containing

only *psiA* cloned in pKT230 also fluoresced under UV light after growth with calcofluor.

Thus, the presence of *psiA* conferred on *R. leguminosarum* the ability to make (or to expose) calcofluor-staining material. To test if this required the formation of the native EPS, a wild-type strain of *R. leguminosarum* bv. *phaseoli* was mutagenized with NTG (see Methods).

Fig. 1. Fluorescing colonies of *psi*-containing strains. Strains of 8401 (to the right in each plate) and 8401(pIJ1393) (to the left) were streaked on TY medium and were photographed under visible (a) and UV (b) light.

Fig. 2. Representation of the *psi* region. Dimensions of the *psiA* and *psiB* genes and location of ORF-P. Restriction sites are for *Pst*I (P) *Bam*HI (B), *Sac*I (S), *Hind*III (H), *Kpn*I (K) and *Sal*I (S). The boxed area above the map represents the previously sequenced *psiA* gene (Borthakur & Johnston, 1987). Various fragments were cloned into Bluescript to form the 'pBIO' plasmids which were sequenced in the orientation indicated. The thick arrow indicates the direction of transcription of the *psi* genes.

Out of several thousand surviving colonies, four were identified which were non-mucoid on TY medium. Each of these was shown to be complemented to EPS⁺ by pBIO139 which carries the *pssA* gene cloned in pKT230 (pBIO139 was made by first cloning a 1076 bp *Eco*RV–*Hind*III fragment containing *pssA* into pUC18 and then releasing *pssA* as an *Eco*RI–*Sac*I fragment before ligating to pKT230). Therefore, these four mutants appear to be affected in the *pssA* gene. One of these mutants (J95) was then used as a recipient for the introduction of pIJ1393 (*psi*). Not surprisingly, the transconjugants were non-mucoid. When plated on TY medium supplemented with calcofluor and examined under UV light, the colonies failed to fluoresce indicating that the production of the calcofluor-staining material in strains containing multi-copy *psi* requires the function of at least one *pss* (*exo*) gene.

Sequencing the *psi* transcriptional unit

Previously (Borthakur & Johnston, 1987), it had been shown that a cloned 1.7 kb *Pst*I–*Eco*RI fragment inhibited EPS production irrespective of its orientation relative to the promoter in the vector and, therefore, this fragment appeared to contain both the *psiA* gene and its promoter. In addition, it contained the gene in which translationally active *phoA* fusions had been identified (Latchford *et al.*, 1991). The DNA sequence of the *psiA* gene had been determined (Borthakur & Johnston, 1987). To complete the sequencing of the region 5' of *psiA* a detailed restriction map was compiled (aided in part by access to sequence information obtained by Borthakur, 1986) and various fragments were cloned into pUC18 prior to sequencing (Fig. 2). Analysis of the region 5' of *psiA* revealed two significant ORFs in the same orientation as *psiA*, both with a high probability of encoding proteins (Fig. 3). The upstream ORF was confirmed to be a gene on the basis of protein fusions (see below) and was termed *psiB*. The intervening ORF was termed ORF-P.

The proposed translational starts of *psiB* and ORF-P were chosen on the basis of their being preceded by satisfactory ribosomal binding site sequences. Analysis of *psiB*–*phoA* fusions shows that the translational start of *psiB* must lie upstream of position 588 (see below). Therefore, *psiB* is likely to start at the GTG(V) at 537 or the TTG(L) at 527. The deduced amino acid sequences of *psiB* and ORF-P were compared with those in databases; no significant similarities to any known sequence were found.

Previously, genetic evidence had shown that the *psi* promoter lies between the *Bam*HI site at position 109 and the *Sac*I site at position 437 (Borthakur & Johnston, 1987). This region was examined for any consensus promoter sequences, regions of dyad symmetry or repeats, but none was found.

Sequencing the *psi*–*phoA* junctions

The recombinant plasmid pIJ1098 contains 30 kb of pRP2JI, including *psiA* and *B*. Derivatives of pIJ1098, containing Tn5–*phoA* insertions which abolished *psi* activity, while expressing AP had been isolated (Latchford *et al.*, 1991). It was noted that on X-P supplemented media, colonies containing the (probably clonal) insertions in pBIO110 and pBIO114 were brighter blue than those in the other three fusion plasmids pBIO113, pBIO115 and pBIO112. To sequence the junctions, the Tn5–*phoA* fusion sites from these five plasmids were subcloned by digesting the plasmids with *Bam*HI (which cuts within Tn*phoA*), ligating the fragments with pUC18 and selecting Kan^r transformants. Such fragments contained the *aph* gene (*kan*), *phoA* plus the part of the *psi* region extending to the next 5' *Bam*HI site.

Using a *phoA*-specific oligonucleotide as a sequencing primer (see Methods) the locations of the *phoA*s were established (Fig. 3). In four cases (two of which – pBIO110 and pBIO114 – are likely to be clonal) *phoA* was

Pst I
1 CTGCAGCATGGGAGATGATTTCGAATTCGCGGGGATTCGTACAGCGTACAGCTCTCGTCAAACCTTGAACACGCACCCGTTTGCATAGCCTGCCCAA 100

BamHI
101 GGTCCGGATCCCGTTCCGCAAAATTTGGGACAAATGTCGAGCCATACGAGCGCGACGCGATTGGGCCGCCACGTTGATTGCTGAACCTCGCCTTAGTCAC 200

201 TGC CGCGGCTGCTGGTATGGTCAACATCGTCGCGTACATGTTTGGCGCGATTGGTATGGGGCGCGCGGATAAACACCGGTATCAATATCTAATAAAGTCA 300

301 ACGATATGGCGACATTGAGCGACACGTCGCGAGCGTGACTTGAATTGAACCGAACCGAATTGGCGCAGGACCATTACACAGCACGCTGAATCTGCATAT 400

Sac I
401 TGCCGCGCGTTTGTGCTATCCCGAGTTGATGACGAGAGCTCTTCGCTCCCGTCAAAGCAGCTGGGCACGGGCTCTTTTCAAGAATTTAGAAGGCAAGGA 500

Sac I pBI0110, pBI0114 >
501 TATGGAATAATTTGTGTATCTGTTGGAGCCGGAAGTGCATCCTTTTCGCGCCGCTGAGCTCCCTGATCGCAACAGCATTGCATCGATCTCCGGTTTAAT 600
RBS V R S F R A A E L P D R N S I A S I S G L I

Hind III
601 TGGCAGTGATCTTATTCAGATGATCCGCTTCGACGACATGCACTCGCTATTTGTTGGGGAAGAGCTTTGCGAGTTGGGTTAACCGCCTTCACGATCTTT 700
G S D L I Q M I R F D D M H S L F V G E E A L R V G L T A F T I F

Kpn I pBI0113 >
701 GACCGGTACCCGATCCCTCTTCCGCGGCGAGATAGCGCTTTTGGGAGGCGACGCTAGTAAACCTTATCGTTCACCGTCAATAACGATGACGGAAGCCGCGC 800
D G Y P I P L A G Q I A L L G G D G S K P Y R S P S I T M T E A A

801 GACGTTTTGAATGTTGCCGCGCGGTGCTTGATCCTGTGTTTGTCTCCGATGGACCGAGTGGCGAACAAGGGCCTCATCGTTGCGGGCGCACTGGAAAGCCT 900
R R F E C C R P V L D P V F A P M D R V A N K G L I V A G A L E S L

pBI0115 > **BamHI**
901 GCAAGTGCATATTGATCGCCGCTCCCGGTGCTCCTATGAGCGCAATGACGAATGATAGGATCCAGGTGCGCGGATTTCGCTCAGAGTCCCGGAGTGTGC 1000
Q V R I D R R S P V L L * RBS

1001 CGACATGGGCTGGAGAAACGGAAGCTGACGCCTCAAAGCGGCGTTCACTACGTTGCGGAAGGCCCTCCGTTCAACTGGACAAGGTCAACACTGGAAGGG 1100
M G W R N G K L T P Q S G V H Y V A E G L P F N W T K V N T E G

1101 CGGAAGACGTTTCAGTGATTTGCCGAGTTGGAAGCCGCGAAGGAAAAAGTCGCCATTTTGCTGTTCGCGCTGACCCGGCTTTTGGCAACCTATGGCACC 1200
R K T F S A F A E L E A A K E K V A I L A V R A D P A F G N L W H

Sal I
1201 AGCTGAATGCCGAAGTAGTTGATCGCTGTCCGACGGCGCAACCCGCTTTGTCATGACAGCGCTGTTTCAACGCCTGCTCTCGGCGAAGTCATGCACGTTTCAT 1300
Q L N A E V V D R C R R R N P L C M Q T L F Q R L L S A K S C T F I

Sal I
1301 AGCGTAGACGAACCGGGCGAAATCATCGGCGTCCGACGAGTTGAAACATTGGACGAACGAACGGGAGCAAGACCTTGCCCTTTAGAACCTTTTTCGAT 1400
A *

pBI0112 >
1401 CCTGCTGCTGCTGTCACGTGACCGAGCTGTTTCGGCCTACGCGCTCGCTTTCCATCTTCAAAGCGTTTCGCGTAACCTTAGCGGCTTCGGTTTTCCTTC 1500
RBS V H Q R C F G L R A S L S I F K A F A V T L A A S V F L

1501 AGGTCGTTTATTTCTTGAGCCTGCTATTATGTCCTTCCGCGCCACGCGGAGAGCGACAGATCGATACATTAGGAACCTCGGCAGGCAGACCAGCCACA 1600
Q V V Y F L S L L F M S F R P T R E S D R S I H S G T R Q A D Q P Q

Hind III **BcoRI**
1601 AAAACGTGATCGCGATAAAACAGAGCAGTCCAACTGCCCAAGCTTGACCAAGCGCTAAACGGCGCACCCCTTAGCGGTGATAGTATTGACCGAATTC 1699
K R D R D K T E Q S N V P K L D P R R K R R T P *

Fig. 3. DNA sequence of the *psi* region. *psiB* extends from 537–936, ORF-P from 1006–1305 and *psiA* from 1420–1670. Relevant restriction sites are shown as are potential ribosomal binding sites (RBS). The locations and corresponding plasmids of the fusions which expressed AP are each marked by a '>'. In all cases, transcription of the *phoA* was from left to right. Potential membrane-spanning regions are shown in bold and the positions of charged amino acids are indicated.

fused in frame with *psiB* and one was in *psiA*. In all cases, the insertions were in the expected orientation.

DISCUSSION

In the work presented here, we have identified and sequenced a gene, *psiB*, upstream of the previously studied *psiA* gene of *R. leguminosarum* bv. *phaseoli*. Between *psiA* and *psiB* is another ORF in the same orientation, but it is yet to be shown formally that ORF-P does represent a

gene. The deduced polypeptide products of ORF-P and *psiB* showed no similarity to any sequences in databases that were interrogated, and there is, at present, no understanding of their biochemical functions.

Insertion mutations into *psiB* and ORF-B have been obtained (Latchford *et al.*, 1991; Borthakur, 1986). Since these insertions would be polar on *psiA*, it is not surprising that their phenotypes mimic those of mutations into *psiA* itself, i.e. loss of the ability to inhibit EPS

synthesis when present in multi-copy plasmids and Fix⁺ on beans when introduced by marker exchange into pRP2JI (Latchford *et al.*, 1991). Thus, the specific effects of non-polar mutations into *psiB* or into ORF-P remain to be established.

Based on the hydrophobic nature of the PsiA protein it had been suggested that it was located in the cytoplasmic membrane of *Rhizobium* (Borthakur & Johnston, 1987). The single 'active' PsiA-AP fusion was at the NH₂-end of PsiA (see Fig. 3) only six amino acids downstream of the Val that was originally suggested by Borthakur & Johnston (1987) to be the NH₂-terminus. In *psiA* there is another potential start codon (TTG) 21 bp upstream of the Val (GTG). In either event this extreme NH₂-region is not itself hydrophobic nor were there any similarities to consensus leader sequences that would translocate the protein into the periplasmic space.

The four *psiB*-*phoA* fusions (two of which are likely to be clonal) were scattered throughout the gene. Analysis of the deduced product of *psiB* revealed the presence of one potential membrane-spanning region near which the two (clonal) insertions were located. Significantly, these two fusions yielded the highest AP activities. It seems likely that this proposed transmembrane domain results in the C-terminal position of PsiB being exposed in the periplasm, but there were no special features in the vicinity of the other two fusions (Fig. 3).

One striking feature that emerged from the studies reported here related to the response of strains with multi-copy *psiA* to the optical brightener calcofluor. As stated, colonies of *R. leguminosarum* do not stain normally with this dye. This is surprising, since calcofluor might be predicted to bind to the glucan backbone of the bacterial EPS. In contrast, the succinoglycan EPS of *R. meliloti* stains brightly with calcofluor when viewed under UV light. Strains of *R. leguminosarum* containing multi-copy *psiA* are non-mucoid (Borthakur *et al.*, 1985) and the supernatants of cultures of such strains contain no detectable EPS (M. A. Jones, unpublished; V. Morris, personal communication). It would seem paradoxical, therefore, that strains with multi-copy *psi* actually stain with calcofluor when the wild-type does not.

We do not know the reason for this phenomenon, but we have speculated that the following mechanism might be responsible. We had suggested that over-production of PsiA caused accumulation of the protein in the inner bacterial membrane and that this somehow jammed the biosynthesis and/or export of EPS (Borthakur *et al.*, 1986). This inhibition can be overcome by presence of multi-copy *psaA*, whose product is also associated with the inner membrane and which may physically interact with PsiA. We tentatively suggest that, although non-mucoid, strains with cloned *psiA* have the capacity to make EPS, but that the (perhaps) nascent molecules may be 'trapped' in the inner membrane and so are not exported. We further suggest that the secondary (or tertiary) structure of these forms of EPS differs from that of the wild-type *R. leguminosarum* such that they can bind

calcofluor, unlike those of the wild-type. It is certainly evident that whatever the identity of the staining material, it requires a functional *psaA* gene, since mutations in *psaA* abolish this phenomenon. These speculations imply that the ability of calcofluor to bind to polymeric glucans and to fluoresce with UV light may depend not only on primary chemical structure, but also to higher orders of molecular structure.

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