Elevated Levels of Synthesis of over 20 Proteins Results after Mutation of the *Rhizobium leguminosarum* Exopolysaccharide Synthesis Gene *pssA*

NELSON GUERREIRO,[1] VLADIMIR N. KSENZENKO,[2]† MICHAEL A. DJORDJEVIC,[1]
TANYA V. IVASHINA,[2] AND BARRY G. ROLFE[1]*

*Genomic Interactions Group, Research School of Biological Sciences, Australian National University,
Canberra City 2601, Australia,[1] and Skryabin Institute of Biochemistry and Physiology of Microorganisms,
Russian Academy of Sciences, Pushchino, Moscow Region 142292, Russia[2]*

The protein expression profiles of *Rhizobium leguminosarum* strains in response to specific genetic perturbations in exopolysaccharide (EPS) biosynthesis genes were examined using two-dimensional gel electrophoresis. Lesions in either *pssA*, *pssD*, or *pssE* of *R. leguminosarum* bv. viciae VF39 or in *pssA* of *R. leguminosarum* bv. trifolii ANU794 not only abolished the capacity of these strains to synthesize EPS but also had a pleiotropic effect on protein synthesis levels. A minimum of 22 protein differences were observed for the two *pssA* mutant strains. The differences identified in the *pssD* and *pssE* mutants of strain VF39 were a distinct subset of the same protein synthesis changes that occurred in the *pssA* mutant. The *pssD* and *pssE* mutant strains shared identical alterations in the proteins synthesized, suggesting that they share a common function in the biosynthesis of EPS. In contrast, a *pssC* mutant that produces 38% of the EPS level of the parental strain showed no differences in its protein synthesis patterns, suggesting that the absence of EPS itself was contributing to the changes in protein synthesis and that there may be a complex interconnection of the EPS biosynthetic pathway with other metabolic pathways. Genetic complementation of *pssA* can restore wild-type protein synthesis levels, indicating that many of the observed differences in protein synthesis are also a specific response to a dysfunctional PssA. The relevance of these proteins, which are grouped as members of the *pssA* mutant stimulon, remains unclear, as the majority lacked a homologue in the current sequence databases and therefore possibly represent a novel functional network(s). These findings have illustrated the potential of proteomics to reveal unexpected higher-order processes of protein function and regulation that arise from mutation. In addition, it is evident that enzymatic pathways and regulatory networks are more interconnected and more sensitive to structural changes in the cell than is often appreciated. In these cases, linking the observed phenotype directly to the mutated gene can be misleading, as the phenotype could be attributable to downstream effects of the mutation.

Soil bacteria belonging to the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Azorhizobium* (collectively termed rhizobia) are able to infect the roots of leguminous plants in a host-specific way and stimulate the formation of nodules. Inside these nodules, rhizobia differentiate into bacteroids that reduce atmospheric nitrogen to ammonia, which is used by the plant. One major characteristic of many rhizobia is the production of large amounts of acidic exopolysaccharide (EPS) molecules which serve a variety of roles in free-living rhizobia and in the establishment of symbiosis.

EPS forms a biofilm layer on the cell surface which is thought to contribute to the following processes: cellular protection against environmental stresses, attachment to surfaces, nutrient gathering, and the preferential absorption of plant secreted flavonoids along the membrane surface (5, 20, 45). In addition, EPS biosynthesis by rhizobia is required for the effective nodulation of legumes such as *Medicago*, *Pisum*, *Trifo-*

lium, *Leucaena*, and *Vicia* spp., which form indeterminate-type nodules (4, 26). EPS-deficient mutants of *Rhizobium leguminosarum* bv. viciae, *R. leguminosarum* bv. trifolii, and *Sinorhizobium meliloti* induce symbiotically defective phenotypes which include delayed root hair curling, nodules devoid of bacteria due to infection threads that abort within peripheral cells of the developing nodule, and small, partially infected, non-nitrogen-fixing nodules (8, 24, 35, 42, 43). The precise function(s) of the EPS molecules in these associations is still unclear; however, recent studies suggest that the strain-dependent variations in chemical structure of these extracellular polymers may enable EPS to function as a symbiotic signaling molecule which regulates plant responses early in the infection process (8, 10, 14, 28, 43).

In general, EPS synthesised by *R. leguminosarum* is a polymer of a conserved octasaccharide repeating unit which is assembled by the sequential transfer of sugars to a growing lipid-linked polysaccharide chain. These repeating units can also be decorated by *O*-acetyl, pyruvyl, and hydroxybutanoyl groups (31, 32). For *R. leguminosarum* strains, the genetic control of synthesis and regulation of EPS is less well understood than in the case of EPSs (EPS I and EPS II) of *S. meliloti* (2, 3, 12, 13, 23). Several genes, designated *pss* genes, have been identified as crucial to the regulation and biosynthesis of EPS in *R. leguminosarum* bv. viciae, *R. leguminosarum* bv. trifolii, and *R. leguminosarum* bv. phaseoli. Functions were as-

---

* Corresponding author. Mailing address: Genomic Interactions Group, Research School of Biological Sciences, Australian National University, G.P.O. Box 475, Canberra City, A.C.T. 2601, Australia. Phone: 61 02 62494054. Fax: 61 02 62490754. E-mail: rolfe@rsbs.anu.edu.au.
† Present address: Institute of Protein Research, Russian Academy of Sciences, Pushchino, Moscow Region 142292, Russia.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference(s) or source
Strains		
R. leguminosarum		
ANU794	Sm^r derivative of *R. leguminosarum* bv. trifolii strain TA1; Nod$^+$ Fix$^+$ on white and subterranean clovers	(7, 35)
ANU437	*pssA*::Tn*5* derivative of ANU794; Sm^r Km^r Exo$^-$ Nod$^+$ Fix$^-$ on white and subterranean clovers	(7, 35)
ANU437/pVF18	ANU437 complemented with pSP329 carrying the *pssA* gene from *R. leguminosarum* bv. viciae VF39; Sm^r Exo$^+$ Nod$^+$ Fix$^+$ on white and subterranean clovers	This study
ANU437/pSPRH4	ANU437 complemented with pSP329 carrying the *pssA* gene from *R. leguminosarum* bv. phaseoli; Sm^r Exo$^+$ Nod$^+$ Fix$^+$ on white and subterranean clovers	This study
VF39	Wild-type *R. leguminosarum* bv. viciae; Rif^r Nod$^+$ Fix$^+$ on *P. sativum*, *V. sativa*, and *V. faba*	(21)
VF39 *pssA*::Tn*5*	*pssA*::Tn*5* derivative of VF39; Rif^r Exo$^-$ Nod$^-$ on *V. faba*	(21)
VF39 *pssA*-Km	Derivative of VF39 containing an insertion of a Km^r cassette within *pssA*; Rif^r Km^r Exo$^{+/-}$ Nod$^+$ Fix$^{+/-}$ on *V. faba*	(25)
VF39 *pssA*::Tn*5*/pVF18	VF39 *pssA*::Tn*5* complemented with pSP329 carrying the *pssA* gene from *R. leguminosarum* bv. viciae VF39; Rif^r Exo$^+$ Nod$^+$ Fix$^+$ on *V. faba*	(21)
VF39 *pssA*::Tn*5*/pSPRH4	VF39 *pssA*::Tn*5* complemented with pSP329 carrying the *pssA* gene from *R. leguminosarum* bv. phaseoli; Rif^r Exo$^+$ Nod$^+$ Fix$^+$ on *V. faba*	This study
VF39 *pssD*::Tn*5*	*pssD*::Tn*5* derivative of VF39; Rif^r Exo$^-$ Nod$^-$ on *V. faba*	(36)
VF39 *pssE*::Tn*5*	*pssE*::Tn*5* derivative of VF39; Rif^r Exo$^-$ Nod$^-$ on *V. faba*	(36)
VF39 *pssC*-Km	*pssC*-Km derivative of VF39; Rif^r Exo$^+$ Nod$^-$ on *V. faba*	(36)
E. coli S17-1	MM294, RP4-2, Tc::Mu-Km::Tn*7* chromosomally intergrated	(39)
Plasmids		
pSP329	RK2ori, Tc^r; derivative of pTJS75 containing pUC18 cloning site	Gift from S. Lory (37)
pSPRH4	Constructed by cloning *pssA* gene from *R. leguminosarum* bv. phaseoli 8002 into pSP329	This study
pVF18	Constructed by cloning *pssA* gene from *R. leguminosarum* bv. viciae VF39 into pSP329	(21)

[a] Nod$^+$, ability to nodulate; Nod$^-$, inability to nodulate; Fix$^+$, ability to fix nitrogen; Fix$^-$, inability to fix nitrogen; Fix$^{+/-}$, reduced ability to fix nitrogen; Exo$^+$, ability to produce EPS; Exo$^-$, inability to produce EPS; Exo$^{+/-}$, reduced production of EPS; Rif^r, rifampin resistant; Sm^r, streptomycin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant.

signed to most of the *pss* genes as a result of genetic complementation or by sequence homology to proteins of known function from other bacteria, including *S. meliloti*. It should be emphasised that the nucleotide sequences of *pss* genes from different biovars of *R. leguminosarum* revealed very high levels of homology, and in most cases they were even identical. Therefore, data obtained with these strains can be considered complementary.

The EPS-deficient mutant strain ANU437, which was used in this study, has been extensively characterized at the phenotypic level. The multiple defects observed suggest pleiotropic effects due to the mutation. In summary, ANU437 is nonmucoid, produces very low levels of acidic EPS (0.3% of that of the parent strain) that lacks an *O*-acetyl substitution, lacks a capsule, is more sensitive to phytoalexins, and induces delayed formation of small, non-nitrogen-fixing nodules on white and subterranean clovers (35). Cellular growth is more sensitive to addition of glycine and succinate than that of the parental strain, and the symbiotic phenotype observed is condition dependent (35). The phenotypes of VF39 *pssA*::Tn*5*, VF39 *pssD*::Tn*5*, and VF39 *pssE*::Tn*5*, which are also used in this study, are somewhat similar to those of ANU437. These strains are also nonmucoid and induce delayed formation of rare non-nitrogen-fixing nodules on *Vicia faba*, but they retain their ability to form nitrogen-fixing nodules on *Pisum sativum* and *Vicia sativa* (21, 36). In contrast, strains VF39 *pssA*-Km and VF39 *pssC*-Km produce EPS at 85 and 38%, respectively, of the wild-type levels. Strain VF39 *pssA*-Km induces nodules on *V. faba* with a reduced ability to fix nitrogen, whereas VF39 *pssC*-Km fails to induce nodules on *V. faba* (25, 36).

The *pssA* gene of *R. leguminosarum* was proposed to encode

a isoprenylphosphate (IP) glycosyl transferase responsible for the first glycosyl transferase step in EPS biosynthesis, involving the transfer of a glucose-1-phosphate residue from UDP-glucose to an IP lipid carrier (6, 21, 33, 41). Interestingly, the *pssA* gene described for *R. leguminosarum* bv. viciae VF39 has an extension of 63 codons at the 5′ end, since translation was found to start at the first putative GTG start codon rather than the second putative start codon as described for *R. leguminosarum* bv. phaseoli (6). Work by Ksenzenko et al. (25) indicated that the *pssA* gene could be expressed and regulated differentially from three different promoters, resulting in two translation variants of PssA, long (263 amino acids [aa]) and short (200 aa). The shorter protein is the glycosyl transferase, whereas the longer protein was postulated to have an additional function(s).

Several other putative glycosyl transferase genes (*pssC*, *-D*, *-E*, *-F*, *-G*, *-H*, and *-I*) from a new gene cluster of *R. leguminosarum* that are involved in EPS biosynthesis have recently been isolated and sequenced (24, 36, 41). The *pssDE* genes are thought to collectively code for a membrane-associated glucuronosyl-(β1→4)-glucosyl transferase that attaches glucuronic acid (GlcA) to Glc-isoprenylpyrophosphate (33). The *pssC* gene probably encodes a glucuronosyl-(β1→4)-glucuronosyl transferase responsible for the third transferase step, involving the addition of GlcA to GlcA-Glc-1P* (33); however, the *pssC* mutant used in this study is still capable of making 38% of the amount of EPS made by the wild-type strain.

Transposon mutagenesis is often used in studies on microbes to identify and sequence genes of interest, investigate the phenotypes resulting from the mutation, and possibly assign function via analogy to homologous genes. These studies can be
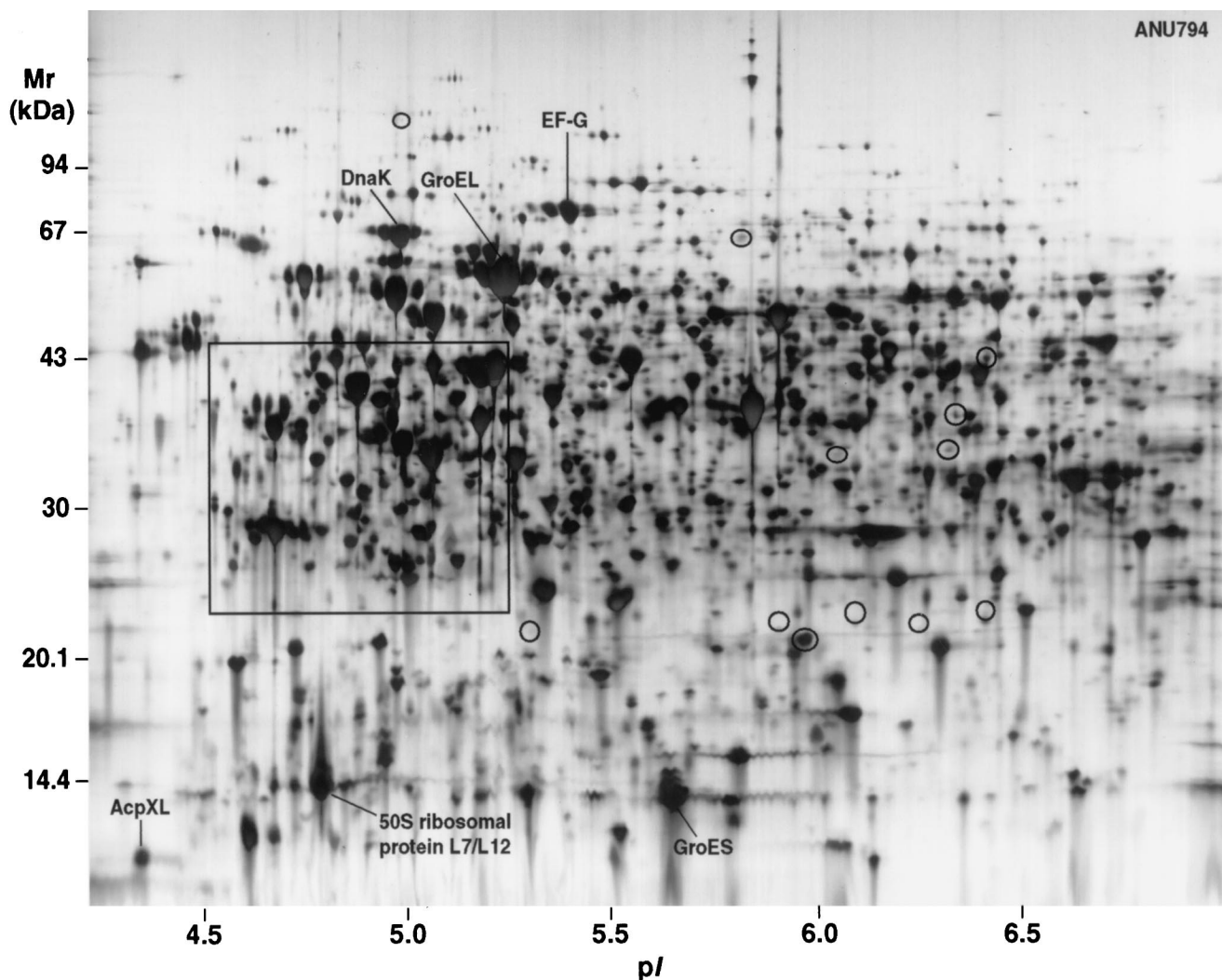
FIG. 1. 2-D protein array of *R. leguminosarum* bv. trifolii strain ANU794. Isoelectric focusing in the first dimension was on IPG strips with a linear gradient ranging from pH 4 to 7 (18 cm) and loaded with 100 μg of total cellular protein. For the second dimension, sodium dodecyl sulfate-polyacrylamide gels (12 to 14% total acrylamide) were used. Proteins were visualized by silver staining. The circled areas correspond to regions on the gel where differences between the wild type and the *pssA* mutant were identified, and these areas are numbered in Fig. 2. The framed area was further resolved using narrow-range IPG strips, and this is shown in Fig. 4.

confounded when pleiotropic effects occur as a result of the mutation. In addition, assigning function in the context of molecular interactions and sequence homology does not always offer a full description of protein function. Protein function needs to be described in the context of higher-order processes such as expression levels and turnover, effects of posttranslational modifications on its activity, involvement in metabolic pathways and signal cascades, and effects of gene knockout or overexpression. The application of global gene expression analysis approaches, such as proteomics, can offer a more complete description of protein function (1, 22). In addition, global gene expression studies have increasingly revealed that single mutations can have unexpected regulatory effects beyond the recognized function of the protein (11).

This study utilized two-dimensional (2-D) gel electrophoresis (2-DE) to examine the responses of various *R. leguminosarum* strains to specific genetic perturbations in the EPS biosynthesis pathway. The proteomes of *R. leguminosarum* bv. trifolii strain ANU794 and *R. leguminosarum* bv. viciae strain VF39 carrying lesions in either the *pssA*, *pssD*, *pssE*, or *pssC* gene, which either abolished or decreased the capacity of these

strains to synthesize EPS, were analyzed for pleiotropic effects caused by the mutation. The aims of this study were to examine the interconnection of the EPS biosynthesis pathway with other cellular functions and to investigate the higher-order processes of protein function.

MATERIALS AND METHODS

Bacterial strains and symbiotic properties. The strains used in this study and their relevant characteristics are listed in Table 1. Since the nucleotide sequences of *pssA* from *R. leguminosarum* bv. viciae (GenBank accession number L48804) and *R. leguminosarum* bv. trifolii (GenBank accession number Y07549) differ only in three positions (substitutions G-2224 for C, T-2226 for G, and T-2228 for A, respectively), all positions are given according to the former sequence. Strain ANU437, a mutant derivative of *R. leguminosarum* bv. trifolii strain ANU794, contains a transposon Tn5 insertion upstream of the second GTG start codon after position 2185, the mutant *R. leguminosarum* bv. viciae VF39 *pssA*-Km contains a Km^r cassette insertion also upstream of the second start codon after position 2060, and the mutant VF39 *pssA*::Tn5 contains a Tn5 insertion within the central part of the *pssA* gene, downstream of the second GTG start codon after position 2359. The symbiotic phenotypes of the *pssA* mutant strains have previously been described (21, 25, 35). The nucleotide sequence of the VF39 *pssE*, *pssD*, and *pssC* genes is presented in GenBank under accession number AF028810. The mutant strains with Tn5 insertions in either *pssD* or *pssE* (after positions 6466 and 6833, respectively) fail to produce EPS and to nodulate *V.*
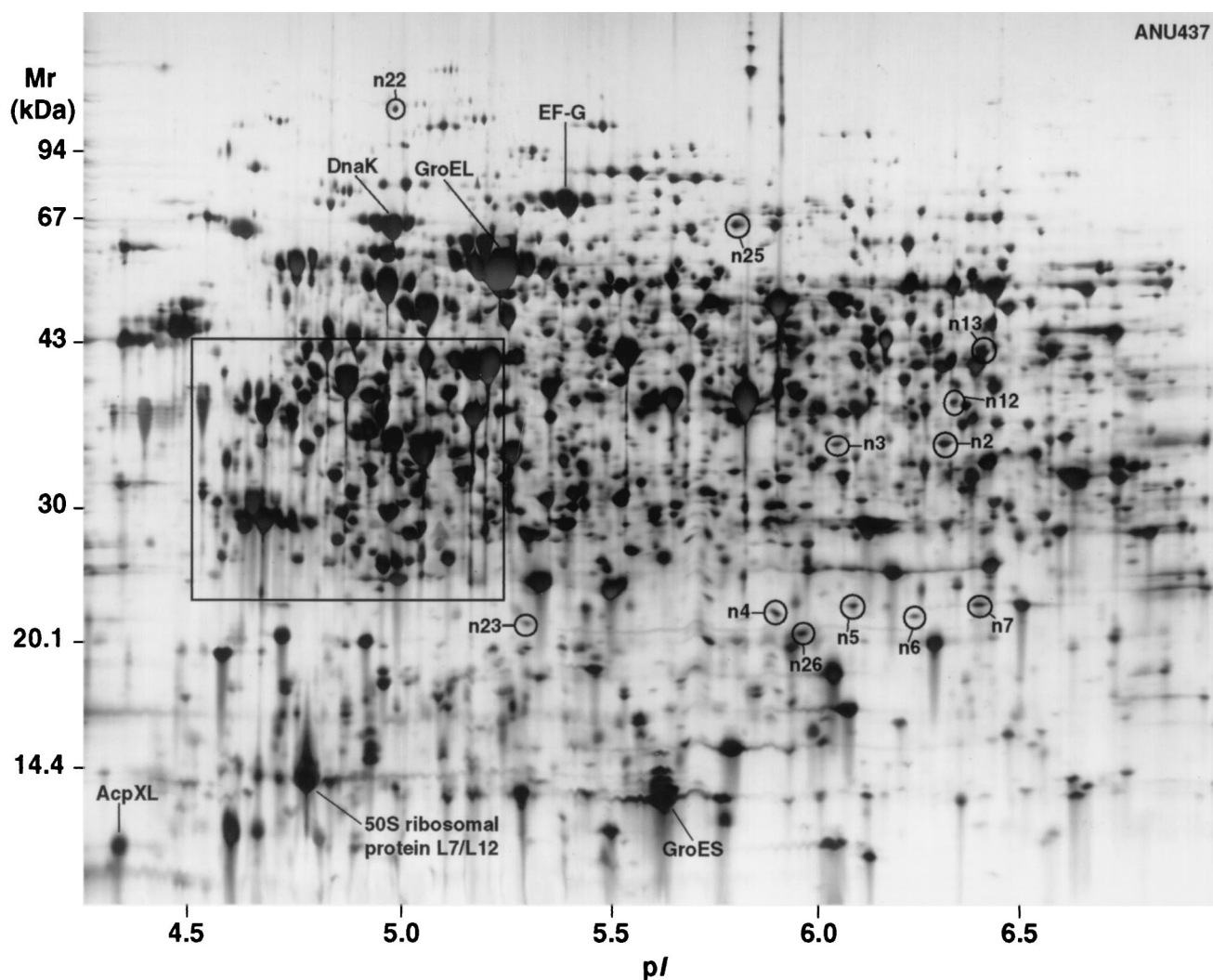
FIG. 2. 2-D protein array of *R. leguminosarum* bv. trifolii strain ANU437 (ANU794 *pssA*::Tn*5*). The protein differences identified between ANU794 and ANU437 are circled and assigned arbitrary numbers. The framed area was further resolved using narrow-range IPG strips, and this is shown in Fig. 4.

faba (36). The strain VF39 *pssC*-Km (a Km^r cassette was introduced into the *Sal*I site after position 5063) produces EPS at levels which are 38% of wild-type levels (36). This strain fails to induce nodules on *V. faba*.

Complementation. Plasmid pVF18, containing the *R. leguminosarum* bv. viciae VF39 *pssA* gene, was described earlier (21). It was constructed by cloning of the *Eco*RI fragment at positions 954 to 4357 into the broad-host-range vector pSP329. Plasmid pSPRH4, containing the *R. leguminosarum* bv. phaseoli *pssA* gene, was constructed by cloning of the *Eco*RV-*Hin*dIII fragment at positions 588 to 1659 of the nucleotide sequence under GenBank accession number X12568. The resulting hybrid plasmids were transferred from the mobilizing strain *Escherichia coli* S17-1 (39) to VF39 *pssA*::Tn*5* or ANU437 strains as described by Simon (38).

Growth conditions and sample preparation. *Rhizobium* strains were grown at 28°C, on a shaker at 200 rpm, in 1 liter of BIII medium (9) containing 2 μg of rifampin per liter (where appropriate) and supplemented with (per liter) 0.2 mg of biotin, 2 mg of thiamine-HCl, and trace elements (0.25 mg of CoCl · 6H$_2$O, 0.25 mg of CuSO$_4$ · 5H$_2$O, 0.25 mg of Na$_2$MoO$_4$ · 2H$_2$O, 3 mg of H$_3$BO$_3$, 3 mg of ZnSO$_4$ · 7H$_2$O, and 10 mg of MnSO$_4$ · 4H$_2$O). During mid-exponential-phase growth (optical density at 600 nm of 0.5 to 0.6) the cells were harvested, washed and lysed as previously described (17).

2-DE and N-terminal sequencing. 2-DE and electroblotting to polyvinylidene difluoride (PVDF) membranes were done by previously described methods (17). Isoelectric focusing in the first dimension was carried out on linear pH 4 to 7 and nonlinear pH 3 to 10 18-cm immobilized pH gradient (IPG) strips (Pharmacia-Biotechnology, Uppsala, Sweden) loaded with 100 μg (0.5 to 1 mg for preparative 2-D gels) of total cellular protein and run for 200 kV · h. N-terminal protein sequencing was done on a PROCISE-HT sequencer system or on a PROCISE-

CLC (both from Perkin-Elmer Applied Biosystems, Foster City, Calif.) for very-low-abundance proteins. Proteins were excised from Coomassie blue-stained PVDF blots (17). N-terminal protein sequences were used to search the nonredundant protein databases (SWISS-PROT, PIR, TREMBL and GenPept) using the FASTA program at URL http://www.angis.su.oz.au/. The N-terminal sequences were also screened against the shotgun sequence database made from *S. meliloti* genomic DNA generated by Melanie Barnett and Sharon Long from Stanford University (see http://cmgm.stanford.edu/~mbarnett/1xgenome.htm or follow the links from http://sequence.toulouse.inra.fr).

Staining and image analysis of 2-D protein arrays. Proteins on analytical 2-D gels were visualized by silver staining (34) and digitized at 600 dots per inch (dpi). Silver-stained 2-D protein arrays were analyzed using the MELANIE II program (Bio-Rad, Hercules, Calif.) for the qualitative and quantitative analysis of differentially displayed protein spots. Internal protein standards were used as pI and M_r reference points (17, 18). Three independent experiments with each mutant strain were performed for the analysis of the differences in protein synthesis. A spot was classified as being differentially displayed if the relative spot volume ratio varied more than twofold between mutants and parental wild-type strains in at least two out of three independent experiments.

RESULTS

Altered levels of expression of several gene products in an *R. leguminosarum* bv. trifolii strain in response to Tn*5*-induced mutation of *pssA*. The proteomes of the EPS mutant ANU437
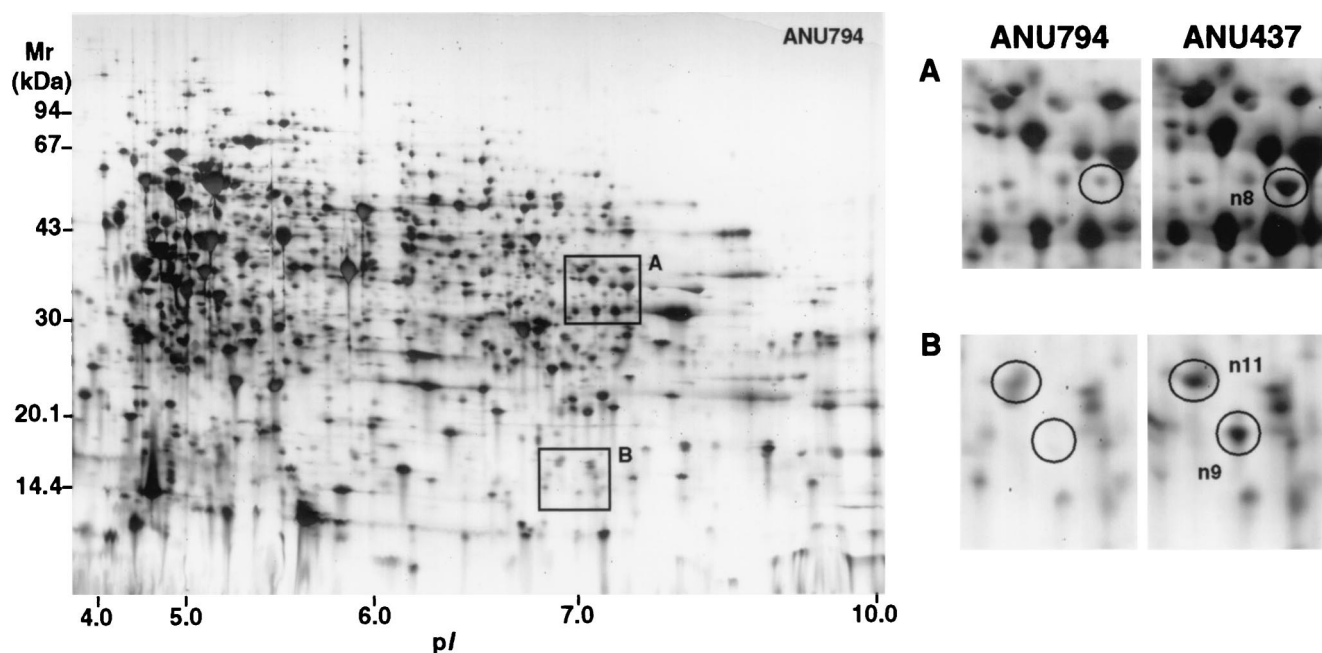
FIG. 3. 2-D protein array of proteins expressed in ANU794 and resolved in the pI range of 3 to 10. The framed areas are enlarged and represent regions on the map where reproducible spot differences were identified between ANU794 and ANU437. The differentially displayed proteins are circled and assigned arbitrary numbers.

and the wild-type parent ANU794 are shown in Fig. 1, 2, and 3. Image analysis revealed over 2,760 soluble proteins of *R. leguminosarum* bv. trifolii, resolved as spots in the pI range of 3 to 10 and size range of 10 to 122 kDa. Twenty-three reproducible differences in the levels of protein synthesis were detected when ANU437 was compared with ANU794. Since these proteins are altered in the mutant strain, they have been termed *pssA* mutant-responsive proteins, and these were grouped as members of the *pssA* mutant stimulon. The *pssA* mutant stimulon consists of 15 up-regulated gene products, designated n2, n3, n8, n11 to n13, n15, n17 to n23, and n25 (Fig. 2 and 3), many of which were grossly up-regulated in their levels of abundance. Seven gene products, designated n4 to n7, n9, n16, and n24, were newly synthesized, and protein spot n26 was down-regulated. Narrow-range IPG strips were used for the first dimension of 2-DE to further resolve the proteins occurring in the pI range of 4.5 to 5.4, where many of the spot differences were located, and these are depicted in Fig. 4. The changes in the relative synthesis levels (relative spot volume ratios) of the *pssA* mutant stimulon proteins are given in Table 2.

Identifying members of the *R. leguminosarum* bv. trifolii *pssA* mutant stimulon. The N-terminal amino acid sequences of 15 members of the *pssA* mutant stimulon were analyzed after they had been electroblotted onto a PVDF membrane and visualized with Coomassie brilliant blue (Table 3). Interestingly, four pairs of newly induced or up-regulated proteins (n4 and n6, n5 and n7, n15 and n21, and n17 and n22) had identical N-terminal sequences. These proteins, except for n17 and n22, most likely represent distinct protein isoforms of similar molecular masses but different pI values. This heterogeneity in charge is likely due to in vivo posttranslational modifications, which can significantly alter the charge on the proteins (46). Interestingly, spots n17 and n22 shared similar pI values, but n22 is approximately four times the size of n17. Spot n22 may represent a protein complex comprised of four n17 subunits,

TABLE 2. Relative differential levels of synthesis of proteins in response to *pssA* mutation in *R. leguminosarum* bv. trifolii ANU437

Spot[a]	% Vol in:		Change in synthesis level[b]			M_r/pI[c]
	ANU794	ANU437	2-fold	3-fold	>5-fold	
n2	0.031	0.080	+			37,436/6.31
n3	0.015	0.037	+			37,368/6.05
n4	0	0.043				22,147/5.90
n5	0	0.035				22,880/6.09
n6	0	0.024				22,147/6.23
n7	0	0.052				22,880/6.38
n8	0.025	0.082		+		34,000/7.50
n9	0	0.056				16,100/7.00
n11	0.028	0.075	+			17,000/6.90
n12	0.023	0.044	+			42,570/6.32
n13	0.034	0.064	+			50,012/6.39
n15	0.064	0.236		+		34,321/5.15
n16	0	0.063				30,566/5.11
n17	0.149	0.273	+			30,456/4.97
n18	0.013	0.096			+	45,109/4.78
n19	0.048	0.154		+		34,633/4.94
n20	0.010	0.057			+	35,467/5.02
n21	0.012	0.078			+	34,197/5.02
n22	0.004	0.025			+	114,862/4.99
n23	0.006	0.026		+		21,632/5.30
n24	0	0.095				31,408/4.64
n25	0.026	0.068	+			74,480/5.80
n26	0.160	0.062	−			20,939/5.96

[a] Arbitrary numbers were assigned to proteins which exhibited a change in their relative synthesis levels in response to a Tn*5* insertion in the *pssA* gene of ANU794.

[b] Relative spot volume ratio for a protein from ANU794 and ANU437 of greater (+) or less (−) than two-, three-, or fivefold.

[c] Observed M_r and pI of the protein based on the migration of proteins in the gels.
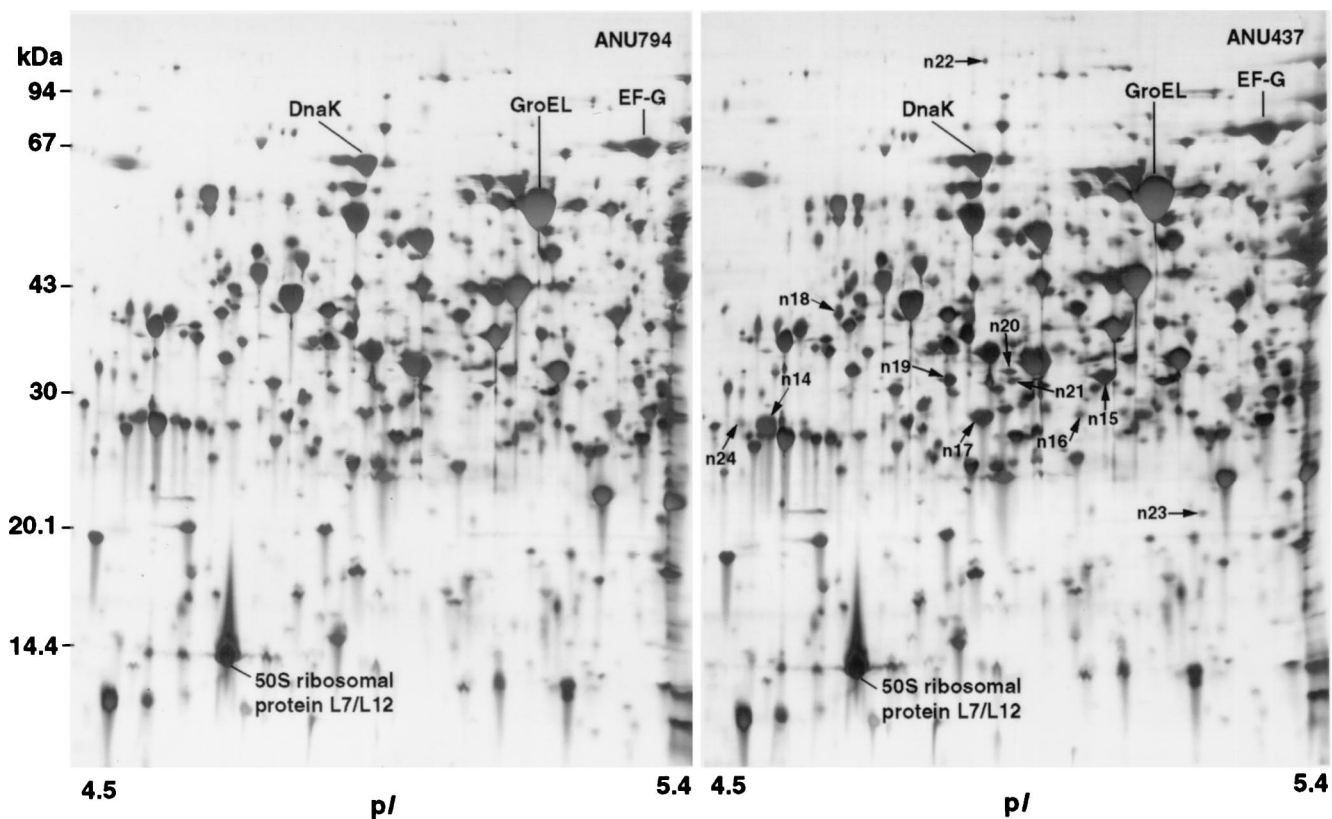
FIG. 4. 2-D gel image representing a protein expression window (proteomic contig) showing proteins from ANU794 and ANU437 in the pI range of 4.5 to 5.4. Narrow-range IPG strips (pH 4.5 to 5.4; 11 cm) loaded with 100 μg of protein were used for the first-dimension separation of 2-DE. The differentially displayed proteins are circled and assigned arbitrary numbers.

which failed to completely dissociate with the 2-D solubilization and separation procedures used.

Sequence alignment searches of the nonredundant protein database revealed that only three protein species exhibited significant sequence homology to previously identified proteins. One differentially displayed protein (n14) was identified as aminoglycoside-3′-O-phosphotransferase, encoded by the Tn5 transposon present in the pssA mutant but not the parental strain (Fig. 4), as expected. Protein spot n2 showed sequence homology to MigA from *Pseudomonas aeruginosa*. MigA is homologous to glycosyltransferases of other gram-negative bacteria that are involved in the biosynthesis of lipopolysaccharides or EPSs (44). The protein isoforms n15 and n21 matched the glutamine-binding periplasmic protein (GlnH) from *E. coli*, starting from the residue Ala-23 of the precursor. It has been shown that a signal peptide of the GlnH precursor consists of 22 N-terminal residues (30). Twelve proteins representing nine protein species did not display any significant sequence homology to proteins currently available in the database. All of the open reading frames of Tn5 are known, so none of the nine species are due to Tn5 proteins. This low success in cross-species homology searching is not surprising considering that rhizobia are poorly characterized at the molecular level and that the mutant-responsive proteins of the pssA mutant stimulon are condition specific.

A further attempt to identify proteins with homology to those altered in abundance in strain ANU437 was done by screening the N-terminal sequences against a shotgun database of the entire *S. meliloti* genome that was translated in all six reading frames (see Materials and Methods). None of the

sequenced proteins showed significant homology to entries in this database.

Pleiotropic alterations in the levels of cellular protein synthesis in *R. leguminosarum* bv. viciae containing a lesion in the *pssA* gene. Proteins synthesized in the *R. leguminosarum* bv. viciae VF39 *pssA*::Tn5 and VF39 *pssA*-Km strains were analyzed by 2-DE and compared to those of the wild-type strain (Fig. 5 and 6). Consistent with the results found with ANU437 and ANU794, 22 reproducible spot differences in the level of synthesized proteins were observed between wild-type VF39 and VF39 *pssA*::Tn5 (Fig. 6). The relative spot volume ratios of these mutant-responsive proteins are given in Table 4. Most of the observed differences involved the up-regulation of gene product expression, while some proteins appeared to be newly synthesized, repressed, or down-regulated in response to the Tn5 mutation. Of the differences observed for VF39 *pssA*::Tn5, only two mutant-responsive proteins exhibited electrophoretic mobilities similar to those observed for ANU437. These similar spot differences are arbitrarily designated n22 and n15 in the protein profile of ANU437 (Fig. 2) and, respectively, v1 and v7 in the protein profile of VF39 *pssA*::Tn5 (Fig. 6). Only two protein differences were observed between VF39 *pssA*-Km and wild-type VF39 (Fig. 5). They involved the induction of two proteins (v26 and v27).

Proteome analysis of the *pssC*, *pssD*, and *pssE* mutant derivatives of *R. leguminosarum* bv. viciae. To examine whether the observed changes in gene expression of the EPS mutants were a result of a dysfunctional *pssA* gene product or due to a response by the cell to the loss of EPS, we analyzed the proteomes of mutants VF39 *pssC*-Km, VF39 *pssD*::Tn5, and VF39
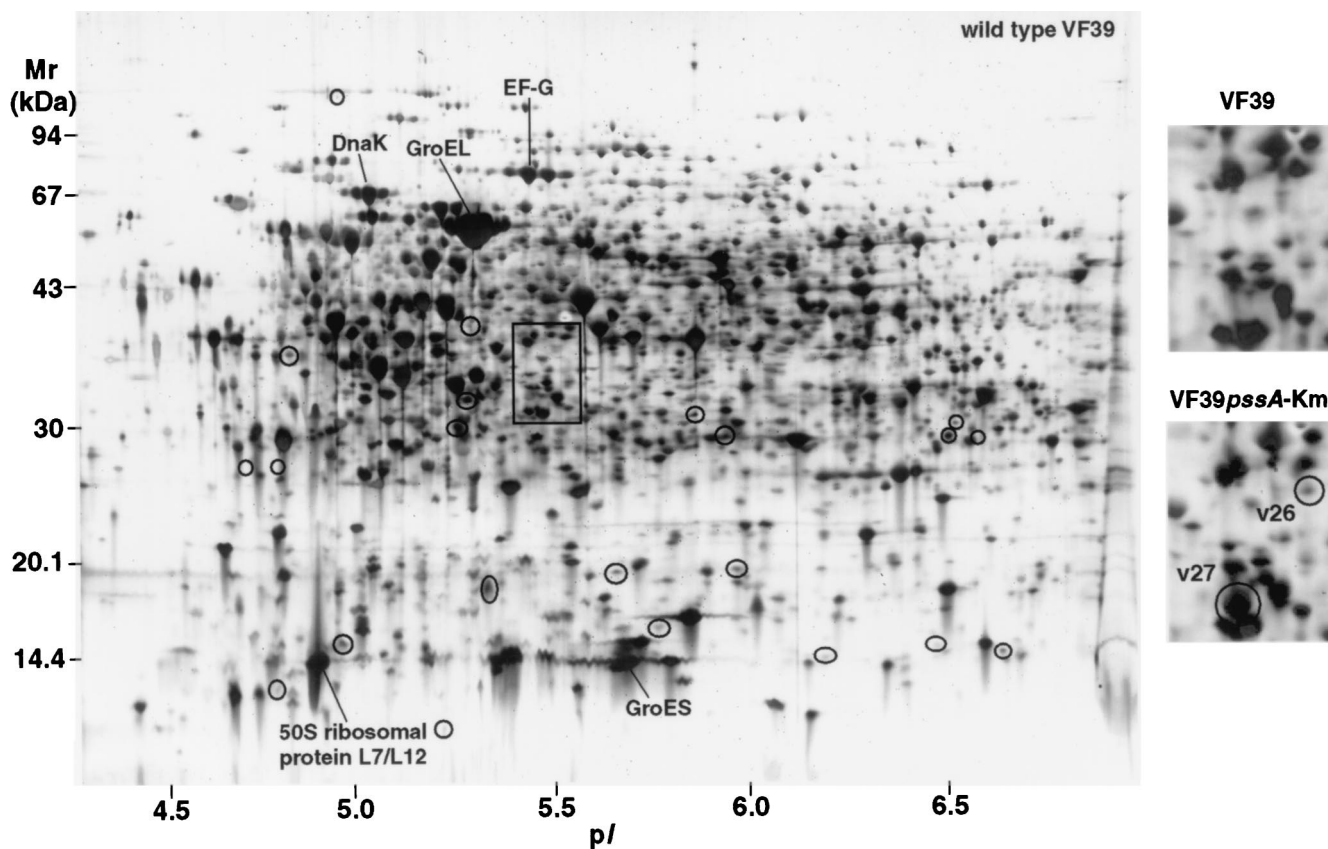
FIG. 5. 2-D protein array of proteins expressed in *R. leguminosarum* bv. viciae strain VF39 and resolved in the pI range of 4 to 7. The circled areas correspond to regions on the gel where differences between the wild type and the *pssA* mutant were identified, and these areas are numbered in Fig. 6. Differences between VF39 and VF39 *pssA*-Km were found only within the framed area, which is enlarged on the right. The spot differences are designated v26 and v27.

pssE::Tn*5*, which carry mutations in putative glycosyl transferases involved in EPS biosynthesis. Of the 22 protein changes associated with VF39 *pssA*::Tn*5*, 9 were also present in the VF39 *pssD*::Tn*5* and *pssE*::Tn*5* mutants at levels similar to those found in the *pssA* mutant. These were spots v2 to v5, v9, v13 to v15, and v20. The other 13 *pssA*::Tn*5*-associated spot changes were present at wild-type levels in the *pssD* and *pssE* mutants. Interestingly, no differences were detected between the mutant VF39 *pssC*-Km and wild-type VF39, except for one induced spot with an electrophoretic mobility similar to that of v27 in the VF39 *pssA*-Km proteome which is, therefore, most likely associated with the Km^r cassette.

Complementation analysis of *pssA* mutants. The plasmids pVF18 and pSPRH4, containing cloned *pssA* genes from *R. leguminosarum* bv. viciae VF39 and *R. leguminosarum* bv. phaseoli 8002, respectively, were transferred into the *pssA* mutants ANU437 and VF39 *pssA*::Tn*5*. In the case of *R. leguminosarum* bv. phaseoli, *pssA* translation starts from the second GTG start codon, leading to a 200-aa-long polypeptide (6). In all four cases, the transconjugants acquired a mucoid morphology comparable to that of the parental strains and were fully complemented for their nodulation-deficient phenotype.

The proteomes of the transconjugants were analyzed to determine the extent to which the wild-type gene can correct the gene expression changes associated with the Tn*5* mutation of *pssA*. 2-D gel comparisons between the ANU437 transconjugants and parental strain ANU794 revealed that all of the protein differences previously observed in ANU437, except for aminoglycoside-3′-*O*-phosphotransferase encoded by Tn*5*,

were completely corrected. In contrast, the VF39 mutants harboring plasmid pVF18 or pSPRH4 were not fully complemented at the protein synthesis level. Most of the differences observed between VF39 *pssA*::Tn*5* and wild-type VF39 were still present, except for spots v1, v5, v9, v10, v13, v15, and v18, which were corrected to wild-type levels in both VF39 transconjugants. The intensity of spot v22 was reduced to a level closer to that of the wild type.

Grouping the mutant-responsive proteins into EPS- and *pssA*-dependent responses. Table 5 shows a comparative analysis of the 22 protein differences identified in the VF39 *pssA*::Tn*5* mutant for the transconjugants and *pssD*, *pssE*, and *pssC* mutants. From the results obtained, the 22 mutant-responsive proteins can be divided into three groups. Group 1 consists of proteins which are responsive to the absence of EPS and include those protein spots whose synthesis levels differed only in strains failing to produce EPS (spots v5, v9, v13, and v15). Group 2 consists of proteins whose wild-type synthesis levels were dependent on the presence of a functional PssA. This group included the protein spots v1, v7, v8, v10, v16 to v19, and v21 to v25. Group 3 consists of proteins which are up-regulated to the same levels in all strains, including *pssA* transconjugants, but are at wild-type levels in the *pssC* mutant (v2 to v4, v14, and v20).

DISCUSSION

Transposon mutagenesis combined with 2-DE-based proteomics has provided an effective approach to analyzing the

TABLE 3. Proteins in the *pssA* mutant stimulon from *R. leguminosarum* bv. trifolii ANU437 analyzed
by N-terminal microsequencing and FASTA searching

ANU437 mutant-responsive proteins			Characteristics of matching protein from database			
Spot[a]	M_r/pI	N-terminal sequence[b]	Homologous protein	Organism	M_r/pI[c]	Swiss-Prot accession no.
n2	37,436/6.31[d]	QTDAPLLSKV :::::.: : TTDAPLVSVV 13 22	MigA	*P. aeruginosa*	34,376/8.31	P95448
n4	22,147/5.90[e]	SNDLIVGFSG	Unknown			
n5	22,880/6.09[e]	SAPKLVGLAGSF	Unknown			
n6	22,147/6.23[e]	SNDLIVGFSGNL	Unknown			
n7	22,880/6.38[e]	SAPKLVGLAGSF	Unknown			
n8	34,000/7.50[d]	SNPVLVNQIIPESRVV [G/T]LT[A/D]SV	Unknown			
n15	34,321/5.15[d]	ADKKVVVAYQTDALP :::::.::: .: .: ADKKLVVATDTAFVP 22 36	Glutamine-binding periplasmic protein	*E. coli*	24,963/6.87	P10344
n16	30,566/5.11[d]	LXXQQKKEFGEFIKQYL	Unknown			
n17	30,456/4.97[d]	EDKSIKVGIMAGEEE	Unknown			
n18	45,109/4.78[d]	DGLSGAPAPFDKGGV	Unknown			
n19	34,633/4.94[d]	TDTVKLRYLASQGNL	Unknown			
n20	35,467/5.02[d]	SILKDDNIDNHADDR	Unknown			
n21	34,197/5.02[d]	ADKKVVVAYQTDALP :::::.::: .: .: ADKKLVVATDTAFVP 22 36	Glutamine-binding periplasmic protein	*E. coli*	24,963/6.87	P10344
n22	114,862/4.99[e]	EDKSIKVGIMAGEEEDIXRVVASEAAK	Unknown			
n23	21,632/5.30[e]	WSTFLYT	Unknown			

[a] Arbitrary numbers were assigned to proteins which exhibited a change in their level of synthesis in response to a Tn5-induced mutation in the *pssA* gene of *R. leguminosarum* bv. trifolii strain ANU794. These numbers correspond to those in Fig. 3, 4, and 5.

[b] N-terminal sequences are shown by using the single-letter for amino acid residues. A comparison of similar sequences is displayed. Colons indicate identical amino acids, and single dots indicate conserved amino acid substitutions.

[d] The predicted molecular weights and isoelectric points of the matched sequences were calculated from the amino acid sequence of the protein using the "compute pI/M.m" tool accessed on the ExPasy server (http://www.expasy.ch).

higher-order processes of protein function and the complex interconnection of the EPS biosynthetic pathway with other cellular networks. A lesion in either *pssA*, *pssD*, or *pssE*, all of which abolish EPS biosynthesis, resulted in a pleiotropic response at the protein synthesis level in *R. leguminosarum* bv. trifolii and *R. leguminosarum* bv. viciae backgrounds. These results demonstrate the complexities of EPS biosynthesis and regulation, which are likely to involve cross talk between regulatory components from different networks, and it is possible that these networks are conserved in these two biovars of *R. leguminosarum*.

The pleiotropic response to *pssA* mutation included the up- and down-regulation, repression, and induction in the levels of synthesis of 22 and 23 proteins, respectively, in the *R. leguminosarum* bv. viciae and *R. leguminosarum* bv. trifolii mutant strains. A polar effect of the mutation has been ruled out as a reason for these changes in protein synthesis, since (i) a rather strong transcriptional terminator has been identified downstream of *pssA*, (ii) an open reading frame situated further downstream is transcribed in the opposite direction (21) (GenBank accession number L48804), and (iii) *pssD* and *pssE* are not transcribed from the same promoter as *pssA*. The members of the *pssA* mutant stimulon represent a functional group, and by further characterizing the members of this group, one can infer more about the function of PssA.

The biological significance of the *pssA* mutant stimulon proteins remains unclear, as the majority of the N-terminal protein sequences obtained from ANU437 show no homology to sequences available in the current databases. Furthermore, several of these proteins were present as distinct isoforms exhibiting identical N-termini and M_rs but different pIs. Two up-regulated proteins, however, showed significant homology at the N terminus to a putative glycosyl transferase, MigA, from the human pathogen *P. aeruginosa*, and to a glutamine-binding periplasmic protein GlnH from *E. coli*. MigA is thought to be involved in the biosynthesis of lipopolysaccharides or EPS in *P. aeruginosa*, and its expression is induced by respiratory mucus derived from individuals with cystic fibrosis (44). Interestingly, *P. aeruginosa* is nonmucoid when first recovered from the respiratory tracts of infected individuals, compared to the normal mucoid phenotype (15), suggesting that MigA is induced in response to signals which regulate the levels of lipopolysaccharide and EPS biosynthesis. The expression of the *R. leguminosarum* bv. trifolii MigA homologue could also be regulated by a similar condition-dependent mechanism, but this requires further investigation. Previous analysis of the extracellular polysaccharides made by ANU437 (35) did not shed light upon a role that this potential MigA homologue might have in the elaboration of an alternative EPS or lipopolysaccharide. GlnH from *E. coli* forms part of ABC-type solute uptake systems that transport glutamine across the cytoplasmic membrane. The expression of *glnD* is significantly up-regulated in *E. coli* cells grown under conditions where glutamine is limiting (29), as would be expected. A possible explanation for the up-regulation of the *R. leguminosarum* bv. trifolii GlnH homologue in ANU437 is that EPS is required for nutrient gathering and concentrating diffusible molecules from the external milieu along the cell surface. The inability to
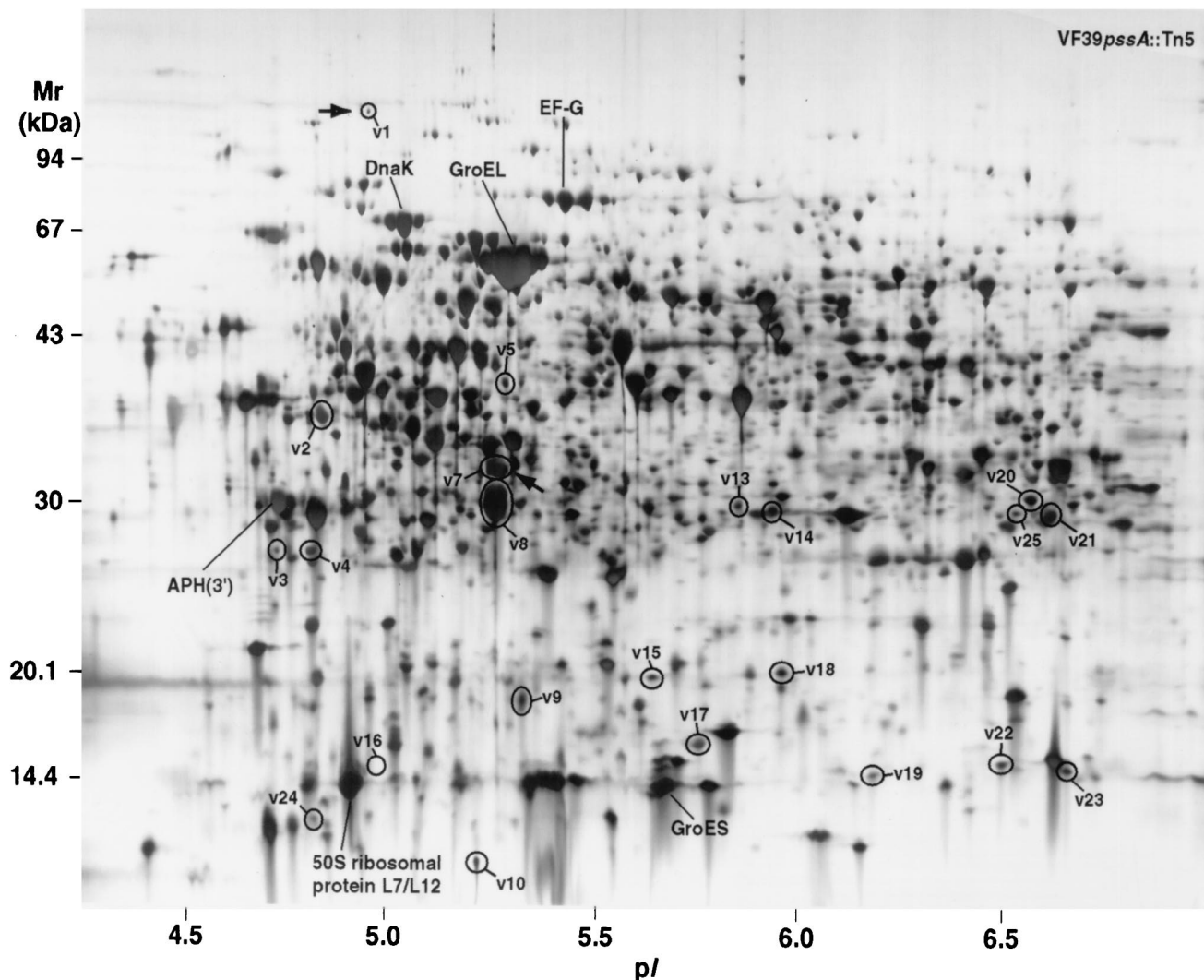
FIG. 6. 2-D protein array of *R. leguminosarum* bv. viciae strain VF39 *pssA*::Tn*5*. A total of 22 protein differences between VF39 and VF39 *pssA*::Tn*5* were identified. The differentially displayed proteins are circled and assigned arbitrary numbers. Arrows point to conserved differences found in both VF39 *pssA*::Tn*5* and ANU437 compared to their respective wild-type strains and are based on the spot electrophoretic mobilities. The arrows correspond to spots n22 and n15 in ANU437. APH(3'), aminoglycoside-3'-*O*-phosphotransferase.

concentrate nutrients on the cell surface of ANU437 may lead to nutrient deficiency and an up-regulation of this gene and its protein product. We could not find any evidence in the literature to directly link GlnH with EPS synthesis.

The mode of *pssA* gene expression in the *R. leguminosarum* biovars trifolii and viciae remains unclear. It is not known whether it is translated in the form of long (263-aa) or short (200-aa) polypeptide. In this study, however, it is clear from the proteomes of the complemented *R. leguminosarum* bv. trifolii ANU437 transconjugants that the putative 63-aa extension does not play a role in the observed protein differences. We deduced this since the *pssA* gene from *R. leguminosarum* bv. phaseoli, which does not have the 63-aa extension, completely restored the proteome of ANU437 to that of the wild type. This is also consistent with the observed proteome for the VF39 *pssA*-Km mutant. In the latter strain, a Km^r cassette was inserted into the *pssA* gene just upstream of the −10 sequence of P3 promoter, in contrast to ANU437, where the Tn*5* insertion was located downstream of this promoter. Therefore, in the case of VF39 *pssA*-Km, one can expect the synthesis only of

the short version of PssA, although at a reduced level, from the altered P3 promoter or a promoter localized in the Km^r cassette. The mode of *pssA* gene expression in rhizobia and the functional significance of a 63-aa extension is currently being investigated using antibodies against PssA (V. N. Ksenzenko and T. V. Ivashina unpublished data).

In contrast to the case for ANU437, complementation of VF39 *pssA*::Tn*5* did not completely restore protein synthesis levels to that of the wild type, even though EPS biosynthesis and nodulation efficiency were restored. The transconjugants are merodiploid, since the mutated *pssA* allele on the genome is genetically complemented rather than genetically replaced and Tn*5* is retained in the complemented strain (the strain retains kanamycin resistance, and the protein product of the kanamycin resistance gene is observed on 2-D gels). The only obvious difference between the two *pssA* mutant strains is the location of the transposon Tn*5* within the *pssA* gene. In the case of VF39 *pssA*::Tn*5*, one can hypothesize the synthesis of a truncated PssA protein, since the Tn*5* insertion is localized 40 codons downstream of the second GTG codon. In the case of

TABLE 4. Relative differential levels of synthesis of proteins in response to *pssA* mutation in *R. leguminosarum* bv. viciae VF39

Spot[a]	% Vol in:		Change in synthesis level[b]			M_r/pI[c]
	VF39	VF39 *pssA*::Tn5	2-fold	3-fold	>5-fold	
v1	0.005	0.011	+			114,000/4.98
v2	0.043	0.181		+		35,178/4.85
v3	0	0.024				26,005/4.74
v4	0	0.100				25,620/4.82
v5	0.013	0.068			+	38,385/5.30
v7	0.074	0.396			+	31,186/5.28
v8	0.335	0.920	+			29,294/5.28
v9	0.086	0.175	+			17,142/5.34
v10	0.007	0.046			+	10,091/5.23
v13	0.033	0.071	+			28,990/5.87
v14	0.057	0.118	+			28,562/5.98
v15	0.028	0.056	+			18,722/5.65
v16	0.060	0				14,093/4.99
v17	0.029	0.111		+		14,793/5.78
v18	0.036	0.091	+			19,047/5.97
v19	0.012	0.042		+		13,500/6.19
v20	0.018	0.127			+	29,557/6.57
v21	0	0.293				28,477/6.62
v22	0.008	0.060			+	14,277/6.48
v23	0.025	0.055	+			13,764/6.67
v24	0.018	0.049	+			11,463/4.83
v25	0.075	0.036	−			28,604/6.53

[a] Arbitrary numbers are assigned to proteins which exhibited a change in their relative synthesis levels in response to a Tn5 insertion in the *pssA* gene of VF39.

[b] Relative spot volume ratio for a protein from VF39 and VF39 *pssA*::Tn5 of greater (+) or less (−) than two-, three-, or fivefold.

[c] Observed M_r and pI of the protein based on the migration of the gels.

ANU437, a truncated protein cannot be synthesised at all if translation starts from the second GTG codon, or only a short 45-aa polypeptide can be synthesized if translation starts from the first GTG codon. The coexpression of a truncated product with the wild-type protein in the transconjugants may interfere with the function(s) of the latter in a well-recognized process called the dominant-negative inhibition effect (19). Indeed, dominant-negative inhibition has been previously observed in genes involved in EPS synthesis (16). This effect has been used to determine protein function and is common to proteins which form multicomponent complexes but has also been observed for monomeric enzymes (27, 40). In most cases, the mutant variants interfere with the functional assembly of wild-type proteins whose activity depends on oligomerization, interfere with the folding pathway of monomeric wild-type enzymes, or act as competitive inhibitors of the wild-type enzyme when substrate is limiting (19, 27). One could speculate that a truncated *pssA* gene product may have an inhibitory effect on the activity of unknown functional domains in the wild-type protein, other than the glycosyl transferase domain, because the merodiploid strain produce EPS and nodulates effectively. This hypothetical truncated protein may affect the levels of expression of a number of proteins involved in other regulatory networks. This phenomenon may explain why full complementation is not observed for the VF39 *pssA*::Tn5 transconjugants. In the case of ANU437, a putative 45-aa polypeptide could be either unstable or insufficient for interaction with wild-type protein compared to the longer truncated protein synthesized in VF39 *pssA*::Tn5. To determine if a dominant-negative effect is occurring, immunochemical analysis of different forms of the PssA in rhizobium cells needs to be carried out, and this work is currently in progress (Ksenzenko and Ivashina, unpublished data).

By comparing the proteomes from the *pssA*, *pssC*, *pssD*, and *pssE* mutants of VF39 along with the proteomes from the VF39 *pssA*::Tn5 transconjugants, the 22 protein differences were grouped into three categories. Mutations in either *pssD* or *pssE* led to the loss of EPS synthesis and to the alteration of 9 of the same 22 proteins affected in the corresponding *pssA* mutant. The 13 other proteins were present at wild-type levels in these two mutants, and these are likely to represent a group of proteins whose synthesis levels change in response to a dysfunctional *pssA* gene product. Together with the dominant-negative inhibition effect observed, these results further suggest a previously unrecognized multifunctional nature of PssA. Others have proposed earlier that the *pssE* and *pssD* genes encode different subunits of the same glycosyl transferase (36). The identical differences in protein synthesis observed for both the *pssD* and *pssE* mutants support this hypothesis. Interestingly, no differences in protein synthesis levels were detected in the case of the *pssC* mutant. From this one can conclude that a complete or nearly complete block in EPS synthesis is needed to induce the observed changes, since reduction of EPS production by more than 60% does not cause any alterations in the VF39 proteome.

A response to the absence of EPS is likely to involve proteins such as the previously mentioned GlnH homologue which are likely to be dependent on the putative functional roles attributed to EPS in nutrient gathering. Alternatively, a block in the EPS biosynthetic pathway, particularly at the *pssA* step, may lead to an increase in the cellular pool of UDP-glucose, which would likely be filtered through to other pathways, and thus an absence of EPS may indirectly affect the levels of expression of proteins involved in these pathways. In addition, Pollock et al. (33) have suggested that the formation and accumulation of incomplete lipid-linked intermediates in EPS mutants may be detrimental largely because the cells are likely not to release the IP carrier from the incomplete repeat structure for other essential cellular functions such as the synthesis of peptidoglycan. It is possible that this disruption provides a secondary signal to disrupt regulatory networks or other metabolic pathways. However, in the *pssD*, *pssE*, and *pssC* mutants the presence of incomplete lipid-linked intermediates in the cells did not appear to further affect protein synthesis, as no differences apart from to those 22 observed for *pssA* were seen and a free IP carrier is available in the *pssA* mutant.

One other group of proteins exhibited elevated synthesis levels in all mutant strains and the transconjugants, except the *pssC* mutant. These proteins are unlikely to be associated with the Tn5 transposon, as they are not present in ANU437 and have M_rs and pIs that differ from those of Tn5-encoded proteins. Sequence information would need to be obtained to investigate the relevance of these proteins. Since these protein differences are absent from the *pssC* mutant, one explanation is that the gene products of *pssA*, *pssD*, and *pssE* may form a functional protein complex, as postulated for EPS production in *S. meliloti* (12), and that the disruption of this complex by mutagenesis or by a truncated *pssA* gene product may lead to the up-regulation of a unique set of proteins.

Studying the proteomes of EPS mutants has become particularly relevant to functional analysis, especially in terms of higher-order processes such as protein-protein interactions and the description of novel networks of interactions. The results from this study have indicated that the *pssA* gene product, in addition to being a glycosyl transferase, may also serve other functions by directly or indirectly influencing the expression of a complex series of unknown genes. The more that is known about the proteins exhibiting the response, the more that can be inferred about the function of PssA. In addition,

TABLE 5. Comparative analysis of the 22 protein differences associated with VF39 *pssA*::Tn5 in the *pssC*, *pssD*, and *pssE* mutants and the VF39 *pssA*::Tn5 transconjugants

Spot no.	Spot difference[a] in:					
	VF39 *pssA*::Tn5[b]	VF39 *pssA*::Tn5/pVF18[c]	VF39 *pssA*::Tn5/pSPRH4[c]	VF39 *pssD*::Tn5[c]	VF39 *pssE*::Tn5[c]	VF39 *pssC*-Km[c]
v1	Up-reg (+++)	0	0	0	0	0
v2	Up-reg (+++)	+++	+++	+++	+++	0
v3	Induced (+++)	+++	+++	+++	+++	0
v4	Induced (+++)	+++	+++	+++	+++	0
v5	Up-reg (+++)	0	0	+++	+++	0
v7	Up-reg (+++)	+++	+++	0	0	0
v8	Up-reg (+++)	+++	+++	0	0	0
v9	Up-reg (+++)	0	0	+++	+++	0
v10	Induced (+++)	0	0	0	0	0
v13	Induced (+++)	0	0	++	++	0
v14	Up-reg (+++)	+++	+++	+++	+++	0
v15	Up-reg (+++)	0	0	++	++	0
v16	Repressed (− − −)	− − −	− − −	0	0	0
v17	Induced (+++)	+++	+++	0	0	0
v18	Up-reg (+++)	0	0	0	0	0
v19	Up-reg (+++)	+++	+++	0	0	0
v20	Up-reg (+++)	+++	+++	+++	+++	0
v21	Induced (+++)	+++	+++	0	0	0
v22	Up-reg (+++)	++	++	0	0	0
23	Up-reg (+++)	+++	+++	0	0	0
v24	Up-reg (+++)	+++	+++	0	0	0
v25	Down-reg (− − −)	− − −	− − −	0	0	0

[a] For comparison, the symbols +++ and − − − are assigned to the spot differences in VF39 *pssA*::Tn5 and represent the maximum degree of up-regulation (up-reg) or repression, respectively. 0 indicates that the synthesis has been restored to a level similar to that of wild-type VF39.
[b] Protein differences compared with wild-type VF39.
[c] Protein differences compared with VF39 *pssA*::Tn5.

the large number of genes whose expression is altered in response to a perturbation in the *pssA* gene highlights the challenges of understanding the higher-order processes of protein function and the complex interconnection of functional networks.

REFERENCES

1. **Anderson, N., and N. Anderson.** 1998. Proteome and proteomics: new technologies, new concepts, and new words. Electrophoresis **19:**1853–1861.
2. **Becker, A., A. Kleickmann, M. Keller, W. Arnold, and A. Puhler.** 1993. Identification and analysis of the *Rhizobium meliloti exoAMONP* genes involved in exopolysaccharide biosynthesis and mapping of promoters located on the *exoHKLAMONP* fragment. Mol. Gen. Genet. **241:**367–379.
3. **Becker, A., A. Kleickmann, H. Kuster, M. Keller, W. Arnold, and A. Puhler.** 1993. Analysis of the *Rhizobium meliloti* genes *exoU*, *exoV*, *exoW*, *exoT*, and *exoI* involved in exopolysaccharide biosynthesis and nodule invasion: *exoU* and *exoW* probably encode glucosyltransferases. Mol. Plant-Microbe Interact. **6:**735–744.
4. **Becker, A., and A. Puhler.** 1998. Production of exopolysaccharides, p. 97–118. *In* H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (ed.), The *Rhizobiaceae*: molecular biology of model plant-associated bacteria, Kluwer Academic Publishers, Dordrecht, The Netherlands.
5. **Beveridge, T. J., and L. L. Graham.** 1991. Surface layers of bacteria. Microbiol. Rev. **55:**684–705.
6. **Borthakur, D., R. F. Barker, J. W. Latchford, L. Rossen, and A. W. Johnston.** 1988. Analysis of *pss* genes of *Rhizobium leguminosarum* required for exopolysaccharide synthesis and nodulation of peas: their primary structure and their interaction with *psi* and other nodulation genes. Mol. Gen. Genet. **213:**155–162.
7. **Chakravorty, A. K., W. Zurkowski, J. Shine, and B. G. Rolfe.** 1982. Symbiotic nitrogen fixation: molecular cloning of *Rhizobium* genes involved in exopolysaccharide synthesis and effective nodulation. J. Mol. Appl. Genet. **1:**585–596.
8. **Cheng, H.-P., and G. C. Walker.** 1998. Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. J. Bacteriol. **180:**5183–5191.
9. **Dazzo, F. B.** 1982. Leguminous root nodules, p. 431–446. *In* R. G. Burns and J. L. Slater (ed.), Experimental microbiol ecology. Blackwell Scientific, Oxford, United Kingdom.
10. **Djordjevic, S. P., H. Chen, M. Batley, J. W. Redmond, and B. G. Rolfe.** 1987. Nitrogen fixation ability of exopolysaccharide synthesis mutants of *Rhizobium* sp. strain NGR234 and *Rhizobium trifolii* is restored by the addition of homologous exopolysaccharides. J. Bacteriol. **169:**53–60.
11. **Fey, S., A. Nawrocki, M. Larsen, A. Gorg, P. Roepstorff, G. Skews, R. Williams, and P. M. Larson.** 1997. Proteome analysis of *Saccharomyces cerevisiae*: a methodological outline. Electrophoresis **18:**1361–1372.
12. **Glucksmann, M. A., T. L. Reuber, and G. C. Walker.** 1993. Family of glycosyl transferases needed for the synthesis of succinoglycan by *Rhizobium meliloti*. J. Bacteriol. **175:**7033–7044.
13. **Glucksmann, M. A., T. L. Reuber, and G. C. Walker.** 1993. Genes needed for the modification, polymerization, export, and processing of succinoglycan by *Rhizobium meliloti*: a model for succinoglycan biosynthesis. J. Bacteriol. **175:**7045–7055.
14. **Gonzales, J. E., G. M. York, and G. C. Walker.** 1996. *Rhizobium meliloti* exopolysaccharides: synthesis and symbiotic function. Gene **179:**141–146.
15. **Govan, J. R. W.** 1988. Alginate biosynthesis and unusual characteristics associated with the pathogenesis of *Pseudomonas aeruginosa* in cystic fibrosis, p. 67–96. *In* E. Griffiths, W. Donachie, and J. Stephen (ed.), Bacterial infections of respiratory and gastrointestinal mucosae. IRL Press, Oxford, United Kingdom.
16. **Gray, J. X., M. A. Djordjevic, and B. G. Rolfe.** 1990. Two genes that regulate exopolysaccharide production in *Rhizobium* sp. strain NGR234: DNA sequences and resultant phenotypes. J. Bacteriol. **172:**193–203.
17. **Guerreiro, N., J. W. Redmond, B. G. Rolfe, and M. A. Djordjevic.** 1997. New *Rhizobium leguminosarum* flavonoid-induced proteins revealed by proteome analysis of differentially displayed proteins. Mol. Plant-Microbe Interact. **10:**506–516.
18. **Guerreiro, N., T. Stepkowski, B. G. Rolfe, and M. A. Djordjevic.** 1998. Determination of plasmid-encoded functions in *Rhizobium leguminosarum* biovar *trifolii* using proteome analysis of plasmid-cured derivatives. Electrophoresis **19:**1972–1979.

19. **Herskowitz, I.** 1987. Functional inactivation of genes by dominant negative mutations. Nature **329:**219–222.

20. **Hubac, C., J. Ferran, D. Guerrier, A. Tremolieres, and A. Kondorosi.** 1993. Luteolin absorption in *Rhizobium meliloti* wild-type and mutant strains. J. Gen. Microbiol. **139:**1571–1578.

21. **Ivashina, T. V., M. A. Khmelnitsky, M. G. Shlyapnikov, A. A. Kanapin, and V. N. Ksenzenko.** 1994. The *pssA* gene from *Rhizobium leguminosarum* bv. *viciae* VF39: cloning, sequence and the possible role in polysaccharide production and nodule formation. Gene **150:**111–116.

22. **James, P.** 1997. Protein identification in the post-genome era: the rapid rise of proteomics. Q. Rev. Biophys. **30:**279–331.

23. **Keller, M., A. Roxlau, W. M. Weng, M. Schmidt, J. Quandt, K. Niehaus, D. Jording, W. Arnold, and A. Puhler.** 1995. Molecular analysis of the *Rhizobium meliloti mucR* gene regulating the biosynthesis of the exopolysaccharides succinoglycan and galactoglucan. Mol. Plant-Microbe Interact. **8:**267–277.

24. **Krol, J., J. Wielbo, A. Mazur, J. Kopcinska, B. Lotocka, W. Golinowski, and A. Skorupska.** 1998. Molecular characterisation of *pssCDE* genes of *Rhizobium leguminosarum* bv. *trifolii* strain TA1: *pssD* mutant is affected in exopolysaccharide synthesis and endocytosis of bacteria. Mol. Plant-Microbe Interact. **11:**1142–1148.

25. **Ksenzenko, V. N., T. V. Ivashina, M. R. Sadykhov, B. M. Chatuev, M. I. Khmelnitsky, A. A. Kanapin, and M. G. Shlyapnikov.** 1994. A role of the *Rhizobium leguminosarum* bv. *viciae pss1* and *pss4* genes in nitrogen fixing symbiosis, p. 142–146. *In* G. B. Kiss and G. Endre (ed.), First European Nitrogen Fixation Conference and Workshop on Safe Application of Genetically Modified Microorganisms in the Environment. Officina Press, Szeged, Hungary.

26. **Leigh, J. A., and G. C. Walker.** 1994. Exopolysaccharides of *Rhizobium*: synthesis, regulation and symbiotic function. Trends Genet. **10:**63–67.

27. **Michaels, J. E., P. Schimmel, K. Shiba, and W. T. Miller.** 1996. Dominant negative inhibition by fragments of a monomeric enzyme. Proc. Natl. Acad. Sci. USA **93:**14452–14455.

28. **Niehaus, K., D. Kapp, and A. Puhler.** 1993. Plant defence and delayed infection of alfalfa pseudonodules induced by an exopolysaccharide (EPSI)-deficient *Rhizobium meliloti* mutant. Planta **190:**415–425.

29. **Nohno, T., and T. Saito.** 1987. Two transcriptional start sites found in the promoter region of *Escherichia coli* glutamine permease operon, *glnHPQ*. Nucleic Acids Res. **15:**2777.

30. **Nohno, T., T. Saito, and J. S. Hong.** 1986. Cloning and complete nucleotide sequence of the *Escherichia coli* glutamine permease operon (*glnHPQ*). Mol. Gen. Genet. **205:**260–269.

31. **Philip-Hollingsworth, S., R. I. Hollingsworth, and F. B. Dazzo.** 1989. Host-range related structural features of the acidic extracellular polysaccharides of *Rhizobium trifolii* and *Rhizobium leguminosarum*. J. Biol. Chem. **264:**1461–1466.

32. **Philip-Hollingsworth, S., R. I. Hollingsworth, F. B. Dazzo, M. A. Djordjevic, and B. G. Rolfe.** 1989. The effect of interspecies transfer of *Rhizobium* host-specific nodulation genes on acidic polysaccharide structure and *in situ* binding by host lectin. J. Biol. Chem. **264:**5710–5714.

33. **Pollock, T. J., W. A. T. van Workum, L. Thorne, M. J. Mikolajczak, M. Yamazaki, J. W. Kijne, and R. W. Armentrout.** 1998. Assignment of biochemical functions to glycosyl transferase genes which are essential for biosynthesis of exopolysaccharides in *Sphingomonas* strain S88 and *Rhizobium leguminosarum*. J. Bacteriol. **180:**586–593.

34. **Rabilloud, T., L. Vuillard, C. Gilly, and J. C. Lawrence.** 1994. Silver-staining of proteins in polyacrylamide gels: a general overview. Cell. Mol. Biol. **40:**57–75.

35. **Rolfe, B. G., R. W. Carlson, R. W. Ridge, F. B. Dazzo, P. F. Mateos, and C. E. Pankhurst.** 1996. Defective infection and nodulation of clovers by exopolysaccharide mutants of *Rhizobium leguminosarum* bv. *trifolii*. Aust. J. Plant Physiol. **23:**285–303.

36. **Sadykov, M. R., T. V. Ivashina, A. A. Kanapin, M. G. Shlyapnikov, and V. N. Ksenzenko.** 1998. Structural and functional organisation of the exopolysaccharide biosynthesis genes in *Rhizobium leguminosarum* bv. *viciae* VF39. Mol. Biol. **32:**665–671.

37. **Schmidhauser, T. J., and D. R. Helinski.** 1985. Regions of broad-host-range plasmid RK2 involved in replication and stable maintenance in nine species of gram-negative bacteria. J. Bacteriol. **164:**446–455.

38. **Simon, R.** 1984. High frequency mobilisation of gram-negative bacterial replicons by the *in vivo* constructed Tn*5*-Mob transposon. Mol. Gen. Genet. **196:**413–420.

39. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.

40. **Singh, S., H. K. Tang, J.-Y. Lee, and G. F. Saunders.** 1998. Truncation mutations in the transactivation region of PAX6 result in dominant-negative mutants. J. Biol. Chem. **273:**21531–21541.

41. **van Workum, W. A. T., H. C. J. Canter Cremers, A. H. M. Wijfjes, C. van der Kolk, C. A. Wijffelman, and J. W. Kijne.** 1997. Cloning and characterisation of four genes of *Rhizobium leguminosarum* bv. *trifolii* involved in exopolysaccharide production and nodulation. Mol. Plant-Microbe Interact. **10:**290–301.

42. **van Workum, W. A. T., A. A. N. van Brussel, T. Tak, C. A. Wijffelman, and J. W. Kijne.** 1995. Ethylene prevents nodulation of *Vicia sativa* sp. *Nigra* by exopolysaccharide-deficient mutants of *Rhizobium leguminosarum* bv. *viciae*. Mol. Plant-Microbe Interact. **8:**278–285.

43. **van Workum, W. A. T., S. van Slageren, A. A. N. van Brussel, and J. W. Kijne.** 1998. Role of exopolysaccharides of *Rhizobium leguminosarum* bv. *viciae* as host plant-specific molecules required for infection thread formation during nodulation of *Vicia sativa*. Mol. Plant-Microbe Interact. **11:**1233–1241.

44. **Wang, J., S. Lory, R. Ramphal, and S. Jin.** 1996. Isolation and characterization of *Pseudomonas aeruginosa* genes inducible by respiratory mucus derived from cystic fibrosis patients. Mol. Microbiol. **22:**1005–1012.

45. **Whitfield, C.** 1988. Bacterial extracellular polysaccharides. Can. J. Microbiol. **34:**415–420.

46. **Wilkins, M. R., J. C. Sanchez, K. L. Williams, and D. F. Hochstrasser.** 1996. Current challenges and future applications for protein maps and post-translational vector maps in proteome projects. Electrophoresis **17:**830–838.