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Characterization of *Rhizobium leguminosarum* Exopolysaccharide Glycanases That Are Secreted via a Type I Exporter and Have a Novel Heptapeptide Repeat Motif

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The *prsDE* genes encode a type I protein secretion system required for the secretion of the nodulation protein NodO and at least three other proteins from *Rhizobium leguminosarum* bv. viciae. At least one of these proteins was predicted to be a glycanase involved in processing of bacterial exopolysaccharide (EPS). Two strongly homologous genes (*plyA* and *plyB*) were identified as encoding secreted proteins with polysaccharide degradation activity. Both PlyA and PlyB degrade EPS and carboxymethyl cellulose (CMC), and these extracellular activities are absent in a *prsD* (protein secretion) mutant. The *plyA* gene is upstream of *prsD* but appears to be expressed at a very low level (if at all) in cultured bacteria. A *plyB*::Tn*5* mutant has a very large reduction in degradation of EPS and CMC. Cultures of *plyB* mutants contained an increased ratio of EPS repeat units to reducing ends, indicating that the EPS was present in a longer-chain form, and this correlated with a significant increase in culture viscosity. Thus, PlyB may play a role in processing of EPS. Analysis of the symbiotic properties of a *plyA plyB* double mutant revealed that these genes are not required for symbiotic nitrogen fixation and that nodulation was not significantly affected. PlyA and PlyB are similar to bacterial and fungal polysaccharide lyases; they contain 10 copies of what we propose as a novel heptapeptide repeat motif that may constitute a fold similar to that found in the family of extracellular pectate lyases. PlyA and PlyB lack the Ca^{2+}-binding RTX nonapeptide repeat motifs usually found in proteins secreted via type I systems. We propose that PlyA and PlyB are members of a new family of proteins secreted via type I secretion systems and that they are involved in processing of EPS.

The *Rhizobium*-legume symbiosis involves an exchange of signalling molecules between plant and bacterium. The main signals produced by the bacterium are lipochitooligosaccharides (Nod factors), which are synthesized by the products of the *nod* genes and are essential for nodulation. The structure and variety of different Nod factors determine the types of legumes that can be nodulated. Some rhizobial strains also secrete proteins that may be involved in signalling during nodulation (13, 14, 23, 44), providing an extension of the Nod factor-based signalling pathway.

The *prsDE* genes encode two components of a type I protein secretion system that is required for the secretion of the nodulation signalling protein NodO and at least three other proteins from *Rhizobium leguminosarum* bv. viciae (16). One of the other proteins secreted via the Prs system is thought to be a glycanase that cleaves the bacterial expolysaccharide (EPS), since a *prsD* mutant lacks the ability to degrade EPS in a plate assay and produces EPS with an increased degree of polymerization (16). EPS is required at an early stage in nodule invasion, and a low-molecular-weight form has been implicated in the infection of roots by *Rhizobium meliloti* (2, 43). However, the mutant of *R. leguminosarum* bv. viciae defective in protein secretion and cleavage of EPS formed fully infected nodules containing bacteroids (16).

Protein secretion via a type I pathway involves a C-terminal, noncleaved secretion signal (46), and, indeed, the C-terminal 24 amino acids of NodO is essential for its secretion (39). No clear consensus sequence exists for these C-terminal secretion signals; their specificity seems to reside in conserved properties of secondary structure (48, 53). Most proteins that are secreted via type I secretion systems (including NodO) contain a characteristic nonapeptide tandem called RTX (repeat in toxin), so called because these repeats are present in many secreted protein toxins. Members of this family of toxins include *Escherichia coli* alpha-hemolysin, *Erwinia chrysanthemi* proteases, *Pseudomonas aeruginosa* alkaline protease, and *Serratia marcescens* protease and lipase. The structures of some proteins carrying this motif have been determined (3, 4). The RTX repeats form a β-roll structure stabilized by Ca^{2+} ions coordinated between adjacent coils of the β-roll. In some cases, the RTX repeats are required for efficient secretion, especially of large, heterologous proteins (12, 25, 39, 46).

The genes encoding type I secretion systems are usually adjacent to the genes encoding the secreted proteins (11, 15, 26, 27, 47), whereas *nodO* is unlinked to the genes required for NodO secretion (37). Genes encoding other proteins secreted by the PrsDE system are unlinked to *nodO*, since they are not present on the symbiotic plasmid pRL1JI (16). Therefore the *R. leguminosarum* bv. viciae Prs system is apparently different from most other characterized type I systems, which usually secrete only a single protein or several very similar proteins that are encoded by genes adjacent to one another.

MATERIALS AND METHODS

Microbiological methods. Rhizobia were grown at 28°C in TY medium (6) with appropriate antibiotics at the following concentrations (micrograms per milliliter): streptomycin, 400; kanamycin, 20; gentamicin, 200; spectinomycin, 20; tetracycline, 10; lividomycin, 5. Where required for the induction of *nod* genes, hesperetin was added to a final concentration of 1 μM. Culture optical densities were measured at 600 nm with an MSE Spectro-Plus spectrophotometer. The bacterial strains and plasmids used are described in Table 1 or the text.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
Strains		
8401	*R. leguminosarum* lacking pSym; Str^r	24
8401(pRL1JI)	Derivative of 8401 carrying the symbiotic plasmid pRL1JI	9
A408	8401 *prsD1*::Tn*5*	16
A412	8401(pRL1JI) *prsD1*::Tn*5*	16
A501	8401(pRL1JI) *plyA2*::Tn*5*	This work
A503	8401(pRL1JI) *plyA1*::Tn*5*	This work
A575	8401(pRL1JI) *prs6*::Tn*5*	This work
A590	8401(pRL1JI) *prsD2*::Tn*5*	This work
A591	8401(pRL1JI) *prsD3*::Tn*5*	This work
A599	8401(pRL1JI) *prsD4*::Tn*5*	This work
A600	8401(pRL1JI) *plyB1*::Tn*5*	This work
A616	Transductant of 8401 with *plyB1*::Tn*5*	This work
A617	Transductant of 8401(pRL1JI) with *plyB1*::Tn*5*	This work
A632	8401 *plyA2*::Tn*5*	This work
A633	8401 *plyA1*::Tn*5*	This work
A636	8401 *plyA*::Spc^r *plyB1*::Tn*5*	This work
A638	8401(pRL1JI) *plyA3*::Spc^r	This work
A640	8401(pRL1JI) *plyA3*::Spc^r *plyB1*::Tn*5*	This work
Plasmids		
pGV910-C1	pGV910 derivative carrying truncated *egl* from *A. caulinodans* on a 4.3-kb *Eco*RI fragment from pRGC1	17
pIJ7298	pLAFR1 cosmid carrying *prsDE* and *plyA*	16
pIJ7349	9.6-kb *Eco*RI fragment from pIJ7298 carrying *prsDE*, in pIJ1891	16
pIJ7419	pIJ7298 derivative with *plyA2*::Tn*5*	This work
pIJ7421	pIJ7298 derivative with *plyA1*::Tn*5*	This work
pIJ7474	16-kb *Eco*RI fragment from pIJ7419 carrying *plyA2*::Tn*5* in pBluescript (SK+)	This work
pIJ7647	pLAFR1 cosmid carrying *plyB*	This work
pIJ7653	pIJ7647 derivative with *plyB1*::Tn*5*	This work
pIJ7708	2-kb *Eco*RI-*Bam*HI fragment with *plyB* from pIJ7647 in pUC18	This work
pIJ7709	2-kb *Eco*RI-*Bam*HI fragment with *plyB* from pIJ7647 in pIJ1891	This work
pIJ7754	12-kb *Eco*RI fragment with *plyA*::Spc^r in pBluescript(SK+)	This work
pIJ7765	Derivative of pJQ200(KS) carrying 12-kb *Eco*RI fragment with *plyA*::Spc^r from pIJ7754	This work
pIJ7871	*plyA* cloned behind a vector promoter in pKT230	This work
pRGC1	pLAFR1 cosmid carrying the *egl* endoglycanase gene from *A. caulinodans*	17

For polysaccharide analysis, bacteria were grown in Y medium (38) containing mannitol (0.2%, wt/vol) as the carbon source. *E. coli* strains were grown in L broth (31), at 37°C. Plasmids were transferred to *E. coli* by transformation and to *Rhizobium* by triparental mating with a helper plasmid. Strains A616 and A617 were made by transduction (8) of 8401 and 8401(pRL1JI) with phage RL38 propagated on the glycanase mutant A600.

Mutagenesis of pIJ7298 was done as follows. An *E. coli* strain carrying pIJ7298 and Tn*5* on the chromosome was grown overnight in an inhibitory concentration of kanamycin (1 mg/ml) to enrich for cells in which Tn*5* had transposed onto pIJ7298. The cells were subcultured in fresh medium containing no kanamycin, and plasmid DNA was isolated. Transformants of *E. coli* carrying Tn*5* in pIJ7298 were selected on tetracycline and kanamycin. Recombination of the Tn*5* mutations into *R. leguminosarum* bv. viciae was done by marker exchange (35).

Nodulation tests were conducted on peas (*Pisum sativum* var. Wisconsin Perfection) with a minimum of 12 plants per test or on vetch (*Vicia hirsuta*), as described by Knight et al. (22), with at least 20 plants per test.

Construction of *plyA plyB* double mutants. To construct strains carrying mutations in both *plyA* and *plyB*, a *plyA3*::Spc^r allele was constructed. The 16-kb *Eco*RI fragment carrying *plyA2*::Tn*5* was cloned from pIJ7419 into pBluescript (SK+) (Stratagene) to form pIJ7474. Digestion of pIJ7474 with *Hpa*I resulted in the loss of an internal Tn*5* fragment that included the kanamycin resistance gene. The 2-kb Spc^r cassette from pHP45Ω (32) was cloned as a *Sma*I fragment into the *Hpa*I-digested pIJ7474 to make pIJ7754. The resulting *plyA3*::Spc^r allele was cloned as an *Eco*RI fragment into the *sacB* suicide vector pJQ200(KS) (33) to make pIJ7765. The double mutants A636 and A640 were made by marker exchange of the *plyA3*::Spc^r allele from pIJ7765 into the *plyB* mutants A616 and A617, respectively. Double mutants were identified as spectinomycin-, kanamycin-, and sucrose-resistant, gentamicin-sensitive colonies.

Protein analysis. For rapid and sensitive detection of secreted NodO, 250 μl of culture supernatant was loaded onto a nitrocellulose membrane (Sartorius) with a slot blot apparatus. The membrane was then immunostained with a mixture of NodO-specific monoclonal antibodies (39) and goat anti-rat immunoglobulin conjugated to horseradish peroxidase (Sigma). The bound complex was visualized with the enhanced chemiluminescence (ECL) kit (Amersham) as specified by the manufacturer.

For analysis of secreted proteins, rhizobia were grown for 24 h at 28°C in TY medium to an optical density of 0.6. Culture-supernatant proteins were concentrated by precipitation with 10% trichloroacetic acid, as described previously (13), except that after precipitation, the trichloroacetic acid was extracted by washing the precipitate with acetone. Proteins from an equivalent of 5 to 10 ml of culture supernatant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7) with 8% acrylamide and visualized by staining with Coomassie brilliant blue R-250.

PlyB was labelled with [^{35}S]methionine by using an *E. coli* T7 RNA polymerase expression system. The *Eco*RI-*Bam*HI fragment carrying *plyB* (see Fig. 1b) was cloned behind the T7 promoter in pT7-5 (40). Expression and labelling with [^{35}S]methionine were carried out in *E. coli* K38(pGP1-2) in the presence of rifampin as described previously (40). Labelled proteins were detected by autoradiography of SDS-PAGE gels. The molecular weight standards used were the broad-range prestained markers from New England Biolabs.

Plate assays for enzyme activities. For detection of glycanase activity, EPS was precipitated from 5-day cultures of strain A408 with 3 volumes of ethanol and redissolved in sterile water. EPS was incorporated into Y agar plates at about 2 mg ml^{-1}. Colonies were grown for 3 days on this medium and then washed off with water. The plates were flooded with 0.1% Congo Red (42) for 15 min and washed with 5% acetic acid. Degradation of EPS was observed as clearings (reduction of staining) under the colony. Carboxymethyl cellulose (CMC) or polygalacturonic acid degradation was detected similarly, except that colonies were grown on Y agar containing 0.1% CMC or polygalacturonic acid instead of EPS. CMC plates were washed for 10 min with 1 M NaCl before being washed with 5% acetic acid.

Isolation of glycanase mutants and complementation by pIJ7647. For the isolation of mutants lacking polysaccharide-degrading activity, Tn*5*-induced mutants were picked onto CMC or EPS agar plates and analyzed for the ability to produce clearings. Colonies which gave reduced degradation were rescreened by the same procedure. For the isolation of a cosmid (pIJ7647) which complemented the *plyB* mutant for activity on CMC agar, an *R. leguminosarum* bv. viciae cosmid library (9) was introduced into A617 (*plyB*). Transconjugants were selected for streptomycin, kanamycin, and tetracycline resistance and were tested for CMC degradation on plates. The Tn*5* mutation from A617 was recombined

onto pIJ7647, to make pIJ7653, by conjugating A617(pIJ7647) with an *E. coli* strain carrying a helper plasmid. *E. coli* transconjugants which contained recombinant derivatives of pIJ7647 carrying the Tn*5* insertion from A617 were selected on L medium containing kanamycin and tetracycline, and the location of Tn*5* was mapped with several restriction endonucleases. The precise location of Tn*5* was obtained by cloning *Eco*RI-*Bam*HI fragments containing each end of the Tn*5* into pUC18 and sequencing from the ends of Tn*5* with an oligonucleotide primer.

Polysaccharide analysis. Culture supernatants were analyzed by the anthrone method (29) to determine the concentration of sugars and by the Lever (28) method to determine the concentration of reducing sugars. Measurements of total sugars were made with reference to a standard mixture of sugars corresponding to the molar ratio of sugar residues present in the bacterial EPS (glucose/glucuronic acid/galactose ratio, 5:2:1) (10). The ratio of EPS repeat units to reducing ends was calculated on the assumption that EPS accounted for all the carbohydrate present in the culture supernatants. An estimate of the molar concentration of the EPS repeat unit was calculated on the assumption that the concentration of the repeat unit is one-eighth of the total estimated sugar concentration. This was then divided by the measured molar concentration of reducing sugars to obtain the ratio of EPS repeat units to reducing ends. The results are presented as the mean values from duplicate experiments (see Table 2). Variation between duplicate experiments was less than 20%. Qualitatively similar results were obtained in several different experiments, although the actual values were variable (probably due to changes occurring during growth), making it difficult to meaningfully average all the results. The viscosities of the cultures were compared by measuring the time taken to flow between marks on a 0.1-ml glass pipette. The readings were normalized against the results for the wild-type strain 8401(pRL1JI). The results are given as the mean values from duplicate experiments and are representative of several experiments (see Table 2). Variation between duplicate experiments was less than 10%. Sugars in the EPS were converted to alditol acetate derivatives and analyzed by gas chromatography (21).

DNA manipulation and sequence analysis. Standard DNA manipulations were carried out by the method of Sambrook et al. (36). A 9.6-kb *Eco*RI fragment, cloned in pBluescript (SK+) (Stratagene) and carrying *plyA*, *prsD*, and *prsE*, was digested with *Nar*I and *Sma*I and religated, resulting in the deletion of 6.2 kb. The remaining 3.4-kb carrying *plyA* and part (809 bp) of *prsD* was cloned into pKT230 as an *Eco*RI-*Sac*I fragment to form pIJ7871. Double-stranded DNA sequencing was carried out on pIJ7708 and on subcloned fragments of pIJ7349 by using exonuclease III deletions to generate templates. Cycle sequencing was done on a Perkin-Elmer PCR 9600 instrument with M13 forward and reverse primers, using ABI "Prism" Dye Primer cycle-sequencing ready-reaction kits. Reactions were run on ABI 377 and 373A DNA sequencers.

Sequence analysis was carried out with the GCG package (version 8; Genetics Computer Group, Madison, Wis.). Homologous proteins in the data library at the National Center for Biotechnology Information were identified with the blastp program (1). Predictions of secondary structure for protein sequences were obtained with the PredictProtein server (34).

RESULTS

Isolation of mutants defective in polysaccharide degradation. Previously we observed that mutation of *prsD* in *R. leguminosarum* bv. viciae abolished the secretion of several proteins and that this correlated with an inability of the *prsD* mutant to cleave EPS and CMC (16). On the assumption that a gene(s) encoding such one secreted protein(s) may be close to *prsD*, we extended the DNA sequence upstream of *prsD* (there was no evidence for such genes downstream of *prsD*). This sequencing revealed an open reading frame (ORF) upstream of and in the same orientation as *prsD* (Fig. 1a). Since the ORF ends 541 bp upstream from *prsD*, it is unlikely to be transcribed as part of the same operon; indeed, a Tn*5* insertion in this ORF (see below) had no polar effect on the secretion of NodO (data not shown), indicating that the mutation is not polar on *prsD*, which is required for NodO secretion. The 450 bp upstream of the ORF is predicted to be noncoding and therefore may include a promoter.

Since the predicted protein sequence encoded by this ORF has significant similarity to that of bacterial and fungal polysaccharide lyases (see below), we thought that it might be responsible for the CMC and/or EPS degradation by *R. leguminosarum* bv. viciae, and we called the gene *plyA*. Two Tn*5* insertions within *plyA* were identified by restriction mapping of mutated derivatives of pIJ7298 (which carries *plyA* and *prsDE*);

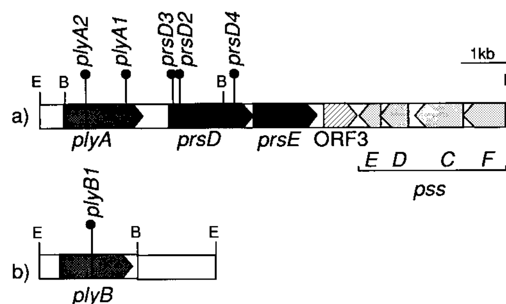

FIG. 1. (a) Map of the 9.6-kb *Eco*RI fragment carring the *prsDE* genes. Downstream from *prsDE* and ORF3 and in the opposite orientation are four genes (*pssFCDE*) homologous to glycosyl transferases in *R. leguminosarum* bv. trifolii (45) and *R. meliloti* (19). Upstream from *prsDE* is *plyA*, encoding a putative polysaccharide lyase. (b) The *plyB* gene is located in a 3.6-kb *Eco*RI fragment. Tn*5* insertions identified in *prsD*, *plyA*, and *plyB* are indicated by solid circles. E, *Eco*RI; B, *Bam*HI.

the Tn*5* insertion sites were confirmed by DNA sequencing from the Tn*5* ends (Fig. 1a). The mutations (*plyA1*::Tn*5* and *plyA2*::Tn*5*) were recombined into *R. leguminosarum* bv. viciae by homologous recombination to make the mutants A632 (*plyA1*::Tn*5*) and A633 (*plyA2*::Tn*5*). Neither mutant was affected in its ability to degrade CMC or EPS in agar plates containing these substrates. This is illustrated for A632 grown on CMC-agar plates (Fig. 2a); as shown, the degradation seen below the A632 colony is not significantly different from that seen with the isogenic control strain (strain 8401). In contrast, degradation of CMC is essentially absent from the *prsD* (protein secretion) mutant A408. These observations showed that the degradation of CMC and EPS observed in the plate assays does not require *plyA* and must be due to a secreted enzyme encoded by another gene.

To identify this other gene, plate assays were used to screen for mutants unable to degrade EPS or CMC. A population of Tn*5*-induced mutants was screened on plates containing either CMC or EPS, and colonies that gave a reduced clearing in comparison with the control strain were identified. This screen was expected to result in the isolation of two classes of mutants: those with mutations in the *prs* genes, which would pre-

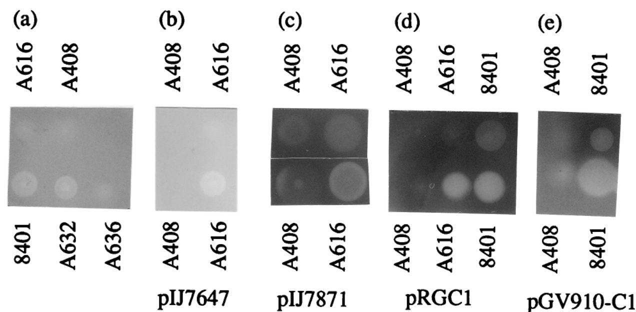

FIG. 2. Degradation of CMC by *ply* mutants. CMC degradation is estimated by the lack of staining (seen as cleared regions) by Congo Red in the agar directly below the colonies. (a) Comparison of the CMC degradation by 8401 (wild-type control), A632 (*plyA*), A616 (*plyB*), A408 (*prsD*), and A636 (*plyA plyB*) on CMC agar. (b) Introduction of pIJ7647 (carrying *plyB*) results in increased degradation of CMC by A616 (*plyB*) but not by A408 (*prsD*). (c) Introduction of pIJ7871 (carrying *plyA*) causes strong degradation by A616 (*plyB*) but not by A408 (*prsD*). (d) Egl from *A. caulinodans*, expressed from pRGC1, causes increased CMC degradation by 8401 and A616 (*plyB*) but not by A408 (*prsD*). (e) Similar results were obtained with Egl expressed from pGV910-C1, except that the CMC degradation extended beyond the diameter of the colony.

vent secretion of the endoglycanase, and those with mutations in a gene encoding a secreted enzyme that degrades CMC and/or EPS. From a total of 12,000 mutant colonies screened, 5 were identified which produced reduced clearing on both EPS and CMC plates. No colonies that degraded only one of the substrates were identified. Genetic complementation studies and mapping of the sites of Tn*5* insertions revealed that four of the five mutations were in *prsD*, and biochemical studies (data not shown) confirmed that these mutants were identical to the *prsD* protein secretion mutant described previously (16). The insertion sites of three of the *prsD* Tn*5* mutations are shown in Fig. 1a.

One mutant was not complemented by the *prsDE plyA* region cloned on pIJ7298. The Tn*5* from this mutant cotransduced (100%) with the defect in CMC and EPS degradation, confirming that a single event caused both phenotypes. The test of CMC degradation by one of the transductants (A616) is illustrated in Fig. 2a. This reveals that there is very little CMC degradation, although a low residual level appears to be present, suggesting that another CMC-degrading enzyme may be present. A similar observation was made with EPS degradation (data not shown). The residual level of CMC degradation by the mutant A616 is highlighted in Fig. 2c, in which the level of staining is enhanced. This shows the presence of a zone of degradation below the A616 colony. There also appears to be a low level of CMC degradation below the *prsD* secretion mutant A408; this may be due to a low level of lysis of some cells in the colony. To determine if the mutation in A616 caused a general effect on protein secretion, the Tn*5* mutation was transduced into a strain carrying the *nodO* gene on the symbiotic plasmid. This mutant (A617) was confirmed to be defective in CMC degradation but retained the ability to secrete NodO (data not shown). This indicates that the Tn*5* mutation affects a gene involved in CMC and EPS degradation rather than a component of the type I protein secretion system.

Identification of *plyB*, which encodes a polysaccharide-degrading enzyme. A cosmid library of *Rhizobium* DNA was crossed into the mutant (A616) defective for CMC and EPS degradation to identify complementing cosmids. The transconjugant colonies were screened for restoration of degradation by using CMC agar plates, and the cosmid pIJ7647 was isolated from one such complemented colony. When pIJ7647 was transferred back into A616, it again restored CMC degradation, demonstrating that pIJ7647 complements the mutation in A616 (Fig. 2b). A 2-kb *Eco*RI-*Hin*dIII fragment subcloned from pIJ7647 could also complement the mutant, and DNA sequencing of this 2-kb fragment revealed a long ORF (1,494 bp). DNA sequencing from the ends of the Tn*5* mutation (cloned from A616) revealed that the Tn*5* is inserted 666 nucleotides downstream of the proposed translation start of the ORF (Fig. 1b). The ORF encoded a predicted protein of 51.5 kDa that is 76% similar (71% identical) to PlyA, and the gene was called *plyB*. In comparison with PlyB, PlyA contains an extra 50 amino acids located 120 residues from the C terminus. Apart from this insert, the similarity extends along the full length of two proteins.

Both PlyA and PlyB show significant similarity to bacterial and fungal polysaccharide lyases, including about 20% identity to PlyD from *Aspergillus niger* (20), PelC from *E. chrysanthemi* (41), and SpsR from *Sphingomonas* strain 888. SpsR may be involved in processing of the sphingan EPS produced by *Sphingomonas* (49). These similarities strongly suggest that *plyB* and *plyA* encode enzymes with polysaccharide-degrading activity; the observation that mutation of *plyB* strongly reduces the degradation of EPS suggests that the primary role of PlyB is the processing of EPS. Since the secretion of CMC and EPS degradation activity is blocked by mutation of *prsD*, we conclude that PlyB is secreted via the *prsDE*-encoded type I secretion system. It is clear that there is little or no degradation of CMC by the *prsD* mutant A408, even when the *plyB* gene is cloned on the multicopy plasmid pIJ7647 (Fig. 2b).

In view of the level of identity between PlyA and PlyB, it seemed likely that *plyA* also encodes an enzyme with polysaccharide-degrading activity. To test this, *plyA* was cloned behind a vector promoter to generate pIJ7871, which was then introduced into the *plyB* mutant (A616), in which the EPS and CMC degradation is greatly reduced. As shown in Fig. 2c, pIJ7871 strongly increases the degradation of CMC, and similar results were seen on EPS-agar plates (not shown). There was no significant CMC (or EPS) degradation when *plyA* (on pIJ7871) was introduced into the protein secretion mutant A408 (*prsD*). On the basis of these results, we conclude that PlyA, like PlyB, is secreted via the *prsDE*-encoded type I secretion system. Such a type I-dependent secretion of PlyA and PlyB is consistent with the absence of potential N-terminal secretion signals in PlyA or PlyB and suggests that they probably have C-terminal secretion signals.

Secretion of an *A. caulinodans* endoglycanase. An endoglycanase gene (*egl*) was previously cloned from *Azorhizobium caulinodans* (17). The *egl* gene product shows no similarity to PlyA and PlyB or to any other proteins shown to be secreted by a type I secretion system. The cloned *egl* gene (on pRGC1) was transferred to A616 (*plyB*) and shown to induce strong degradation of CMC (Fig. 2d). However, there was no degradation of EPS by this strain (data not shown), demonstrating that although the *egl* gene product can degrade CMC, it cannot degrade the EPS from *R. leguminosarum* bv. viciae.

The observation (Fig. 2d) that the cloned *egl* gene does not cause CMC degradation in the protein secretion mutant A408 (*prsD*) demonstrates that Egl secretion is *prsD* dependent. Geelen et al. (17) described a derivative of the *egl* gene that retained CMC degradation activity even though a large part of the gene encoding the N-terminal domain of the Egl protein was deleted. This deleted form of the Egl protein could be also secreted by *R. leguminosarum* bv. viciae, as observed by the strongly enhanced degradation of CMC by *R. leguminosarum* bv. viciae 8401 carrying the deleted form of *egl* (on pGV910-C1). This demonstrates that the deleted N-terminal region of Egl is not required for its secretion. The observed secretion is PrsD dependent as judged by the lack of CMC degradation by the *prsD* mutant A408 carrying the deleted form of *egl* (Fig. 2e).

The pattern of CMC degradation by the strain carrying the deleted *egl* gene was different from that observed in all of the other assays. In all the other cases, the zone of CMC degradation was observed only directly below the colony; i.e., there was no observed diffusion of CMC degradation activity beyond the edge of the colony. However, with the deleted derivative of *egl* (on pGV910-C1), the zone of CMC degradation extended well beyond the edge of the colony (Fig. 2e). The wild-type form of Egl was shown to be cell associated in *A. caulinodans* (17), and this also seems to be the case in *R. leguminosarum* bv. viciae with wild-type Egl, which does not diffuse beyond the edge of the colony (Fig. 2d). It may be that this cell association is mediated via the N-terminal half of the protein and that deletion of this region allows a more freely diffusible activity.

Analysis of culture-supernatant proteins of *ply* mutants. Previously we identified three proteins (other than NodO), with M_rs of 33,000, 65,000, and 110,000, which were not secreted by a *prsD* mutant. To determine which (if any) of these proteins was absent from *plyA* or *plyB* mutants, culture supernatant proteins from 8401 (wild type), A616 (*plyB1*::Tn*5*),

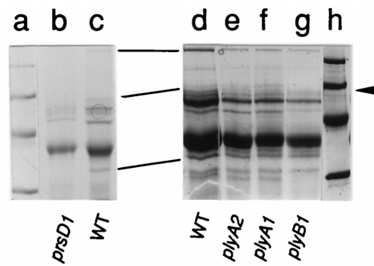

FIG. 3. Two Coomassie blue-stained SDS-PAGE gels showing secreted proteins from *R. leguminosarum* bv. viciae mutants. Culture supernatant proteins were precipitated from the *prsD* mutant A412 (lane b), wild-type (WT) 8401(pRL1JI) (lanes c and d), the *plyA* mutants A501 (lane e) and A503 (lane f), and the *plyB* mutant A617 (lane g). Molecular size markers (94, 67, 43, and 30 kDa) are shown in lanes a and h. The strains were grown in the absence of the *nod* gene inducer hesperetin. Proteins present in 8401(pRL1JI) but absent from the secretion mutant A412 are indicated in lanes c and d. One of these proteins (arrowhead) is apparently also absent from A617 (lane g). The gel containing lanes a, b, and c was run slightly differently from the other gel; the lines highlight the positions of the proteins that were absent from the supernatant of *prsD* mutants.

A632 (*plyA2*::Tn*5*), and A633 (*plyA1*::Tn*5*) were concentrated, separated by SDS-PAGE, and stained with Coomassie blue (Fig. 3). No bands were absent from the culture supernatants of the *plyA* mutants; the bands previously identified as being secreted via PrsDE were all present, indicating that none of these is encoded by *plyA*. A band running at about 65 kDa was absent from (or significantly reduced in amount in) the culture supernatant of the *plyB* mutant A616 (Fig. 3, lane g). This band corresponds to one of the proteins previously observed to be missing from the culture supernatant of a *prsD* mutant (Fig. 3). However, the predicted molecular mass of PlyB (51.5 kDa) is significantly lower than the apparent molecular mass of the missing protein band (65 kDa). The only other difference compared with the control concerns a protein of 30 kDa, whose was reduced in the supernatant of the *plyB* mutant (Fig. 3, lane g), but this decrease was more variable between different preparations. We considered it possible that the PlyB protein migrated aberrantly on SDS-PAGE. *plyB* was expressed in *E. coli* by using a T7 expression system in which expressed proteins were labelled with [^{35}S]methionine (40). A *plyB*-specific band of 52 kDa was detected following SDS-PAGE and autoradiography (data not shown). Therefore, we conclude that PlyB migrates normally on SDS-PAGE and that we are unable to detect its absence in the culture supernatant of the *plyB* mutant. The absence of the 65-kDa protein from the Coomassie blue-stained gel of the *plyB* mutant may be due to a secondary effect (such as lack of expression of a downstream gene) in the *plyB* mutant.

Analysis of EPSs of *ply* mutants. The *prsD* mutant was found to have an increased ratio of total to reducing sugars in culture supernatants and an increase in culture viscosity over the wild-type strain (16). We analyzed the effects of the *plyA* and *plyB* mutations on the EPS by using the same methods that we previously used to characterize the EPS of the *prsD* mutant. Parallel experiments were done with the mutants containing or lacking the symbiotic plasmid pRL1JI, which was found to have no effect on the results. Therefore, for simplicity and to enable a direct comparison with our previous data (which was obtained with pRL1JI-containing strains), we present only the results of the analysis of EPS in the *plyA* and *plyB* mutants carrying pRL1JI.

The amount of EPS (determined by measuring the total sugar content by the anthrone method) made by the *plyA* mutant (A501) was similar to that observed with the control strain [8401(pRL1JI)], and there was also no observed difference in the concentration of reducing sugars (an estimate of the numbers of free ends of EPS) (Table 2). Therefore, the calculated ratio of EPS repeats to reducing sugars is similar to that in the control, 8401(pRL1JI). The viscosity of cultures of A501 (*plyA*) was not increased. These observations indicate that the *plyA* mutation does not greatly affect EPS processing, at least under the growth conditions used. The *plyB* mutant had a slightly higher level of EPS, since the total sugar content was slightly higher than that of the control [8401(pRL1JI)] (Table 2), but the concentration of reducing sugars was below the limits of sensitivity of the assay. Under the conditions used, the assay could detect reducing sugars at concentrations above 0.2 μg ml^{-1}. Therefore, the level of reducing sugars found with the *plyB* mutant (A617) was less than 1/10 of the level seen with the control [8401(pRL1JI)]. On this basis, the ratio of EPS repeats to reducing ends in the *plyB* mutant was calculated to be more than 100 times that observed with the wild-type. The measurements of viscosity supported these results; A617 (*plyB*) cultures were twice as viscous as control [8401(pRL1JI)] cultures after 5 days of growth (Table 2), indicating that the EPS of the mutant is longer than normal. Even when the culture supernatants were adjusted to have similar carbohydrate concentrations, the viscosity of the *plyB* mutant was much higher (about 1.8- to 2-fold) than that of the wild type. There is a theoretical possibility that these altered characteristics of the *plyB* mutant are due to the formation of a new EPS. However, gas chromatography analysis of alditol acetate derivatives of culture supernatant polysaccharides from the *plyB* mutant (and the *plyA* mutant) demonstrated that the ratio and amount of neutral monosaccharides were the same as in the wild-type control (data not shown). Therefore, the increase in culture viscosity and estimated polysaccharide length observed in the *plyB* mutant is unlikely to be due to the formation of a novel high-molecular-weight polysaccharide.

Analysis of the role of *plyA* and *plyB* in the symbiosis. The protein secretion mutant A412 (*prsD*), which cannot secrete NodO, PlyB, PlyA, or other proteins, induces Fix$^-$ nodules on peas. As is typical of Fix$^-$ mutants, this was accompanied by an increase in nodule number from an average of about 80 to about 120 nodules/plant (16). This phenotype cannot be explained by the absence of secreted NodO, since nodules induced by a strain lacking *nodO* are Fix$^+$ (14). One or more proteins that are secreted via the PrsDE system are presum-

TABLE 2. Analysis of EPSs

Strain	Relative culture viscosity	Amt of carbohydrate in culture supernatant (μg/ml)	Amt of reducing sugars in culture supernatant (μg/ml)	Ratio of EPS repeat units to reducing ends
8401(pRL1JI)	1.0	1,800	2.7	82
A412 (*prsD*)	2.7	2,400	<0.2	>1,200
A632 (*plyA*)	0.65	2,100	2.5	100
A616 (*plyB*)	2.0	2,900	<0.2	>1,425

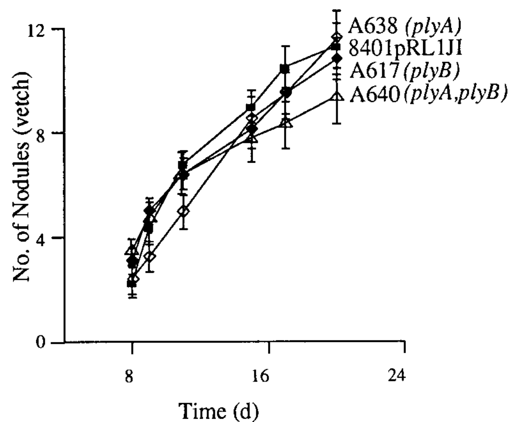

FIG. 4. Nodulation of vetch by 8401 pRLIJI (■), the *plyA* mutant A638 (◇), the *plyB* mutant A617 (◆), and the double mutant A640 (*plyA plyB*) (△). Standard errors are shown.

ably required for the development of an effective symbiosis. The number of nodules induced on peas by A638 (*plyA*) and A617 (*plyB*) was normal (an average of about 80 nodules per plant after 24 days of growth), and this correlated with normal levels of nitrogen fixation (based on measurements of acetylene reduction). The possibility remained that *plyA* and *plyB* were fulfilling a similar function and that either one of them was sufficient for nitrogen fixation; therefore, a double mutant was constructed. The *plyA plyB* double mutant (A640) was also normal with respect to the number of nodules formed and levels of symbiotic nitrogen fixation, demonstrating that PlyA and PlyB are not required to establish a nitrogen-fixing symbiosis. The rate of nodulation of the various mutants on vetch was measured (Fig. 4). A640 (*plyA plyB*) induced slightly fewer nodules than did the control [8401(pRL1JI)], A638 (*plyA*), and A617 (*plyB*). This indicates that *plyA* and *plyB* have little influence on the efficiency of nodulation.

Since *plyB* mutants retain a small residual activity on CMC plates and EPS plates, another enzyme which is able to degrade CMC and EPS may be present. Mutant strains lacking both *plyA* and *plyB* retained the same level of residual activity seen with the *plyB* mutant on CMC agar and EPS agar. The residual activity in the *plyB* mutant is therefore not dependent on PlyA and may be due to an additional polysaccharide-degrading enzyme(s) secreted by *R. leguminosarum* bv. viciae. The *plyA plyB* double mutant was also indistinguishable from the *plyB* mutant in terms of culture viscosity and supernatant polysaccharides (data not shown).

Structural characteristics of PlyA and PlyB. Our data suggest that PlyA and PlyB cleave EPS and depend on the *prsDE* genes for their secretion. The C-terminal 70 amino acids of PlyA and PlyB are very similar and are expected to contain a secretion signal. NodO, PlyA, PlyB, and Egl, all proposed to be secreted by PrsDE, have a characteristic motif at the extreme C terminus. This is made up of a negatively charged residue followed by several hydrophobic residues and is often present in proteins secreted via type I secretion systems (18).

Another characteristic shared by most of the proteins secreted via type I secretion systems is the presence of glycine- and aspartate-rich nonapeptide RTX repeats (46), which form a Ca^{2+}-binding β-roll structure (3, 4). NodO (30 kDa) contains 12 such RTX repeats (13). PlyA and PlyB do not contain an RTX domain, and there is no sequence similarity between NodO and PlyA or PlyB, which are secreted via the same (PrsDE) secretion system. However, we noted that PlyA and PlyB each contain 10 repeats of a novel motif with the approximate consensus N(I/V)X(I/V)X(D/E)N (Fig. 5a). Predictions of secondary structure for PlyA and PlyB suggest that they are composed predominantly of β-strand, particularly in the regions containing the proposed novel repeats (Fig. 5b). Six similar repeats are present in SpsR (Fig. 5a), which has about 20% identity to PlyA and PlyB.

The crystal structures of PelC and PelE from *E. chrysanthemi* (which are similar to PlyA and PlyB) have been solved (50, 51). Both form a parallel β-helix, with three β-sheets, PB1, PB2, and PB3, stabilized by specific stacking interactions between amino acid side chains in the core of the helix. The prediction of secondary structure for PlyA and PlyB was obtained with PHDsec (34) and was superimposed onto a sequence alignment of PlyA and PlyB with PelC. The predicted secondary structure aligned well with the known PelC structure (Fig. 5b). The repeat motifs tended to align with the β-strands forming the PB2 β-sheet. In particular, the Asn residues involved in the formation of a distinctive "asparagine ladder" between PB2 and PB3 in pectate lyases are conserved in PlyA and PlyB (Fig. 5b). These conserved asparagines often correspond to the last residue of the repeat motif described above. Another feature of the PelC structure is the presence of an N-terminal α-helix that caps the β-helix. PlyA and PlyB are predicted to form an α-helix in a similar position (around residue 30). Therefore, rather than forming a β-roll structure based on RTX repeats like other proteins previously shown to be secreted by type I systems, PlyA and PlyB may share a similar fold with the superfamily of extracellular pectate lyases.

In view of the apparent structural similarities between PlyA or PlyB and pectate lyases, we checked for hydrolysis of polygalacturonic acid, which has been seen previously in *R. leguminosarum* bv. trifolii (30). *R. leguminosarum* bv. viciae produced clearings on agar plates that contained polygalacturonic acid, but this activity was not dependent on *plyA* or *plyB*, since isogenic strains carrying mutations in either or both genes had similar clearings to the control strain, 8401 (data not shown). Therefore, although PlyA and PlyB are suggested to form a similar structure to the family of pectate lyases, they apparently do not have the same substrate specificity as this family.

DISCUSSION

Two genes, *plyA* and *plyB*, which are very similar to each other and encode products that are similar to polysaccharide lyases have been identified. PlyA and PlyB are secreted by the Prs protein secretion system encoded by *prsDE* and cleave EPS and CMC. The lack of secreted PlyB can account for the increase in the length of EPS chains observed in the *prsD* mutant.

Some of the proteins secreted via the *prsDE* system may remain cell associated. Cellulase and endoglycanase activities in *R. leguminosarum* bv. trifolii and *A. caulinodans* were found to be cell associated (17, 30). Our results also suggest that Egl is secreted via the PrsDE type I secretion system but may remain associated with the cell surface. Such an association could account for the lack of diffusion of CMC-degrading activity from colonies and could be somewhat different from the secretion of soluble proteins such as NodO, proteases, and alpha-hemolysin secreted via type I exporters. *R. leguminosarum* bv. trifolii, which had a cell-associated extracellular cellulase activity, had no cellulase activity in culture supernatants (30). Similarly, the Egl endoglycanase from *A. caulinodans* was also concluded to be a cell-associated extracellular enzyme (17). The diffusion of enzyme activity from colonies carrying

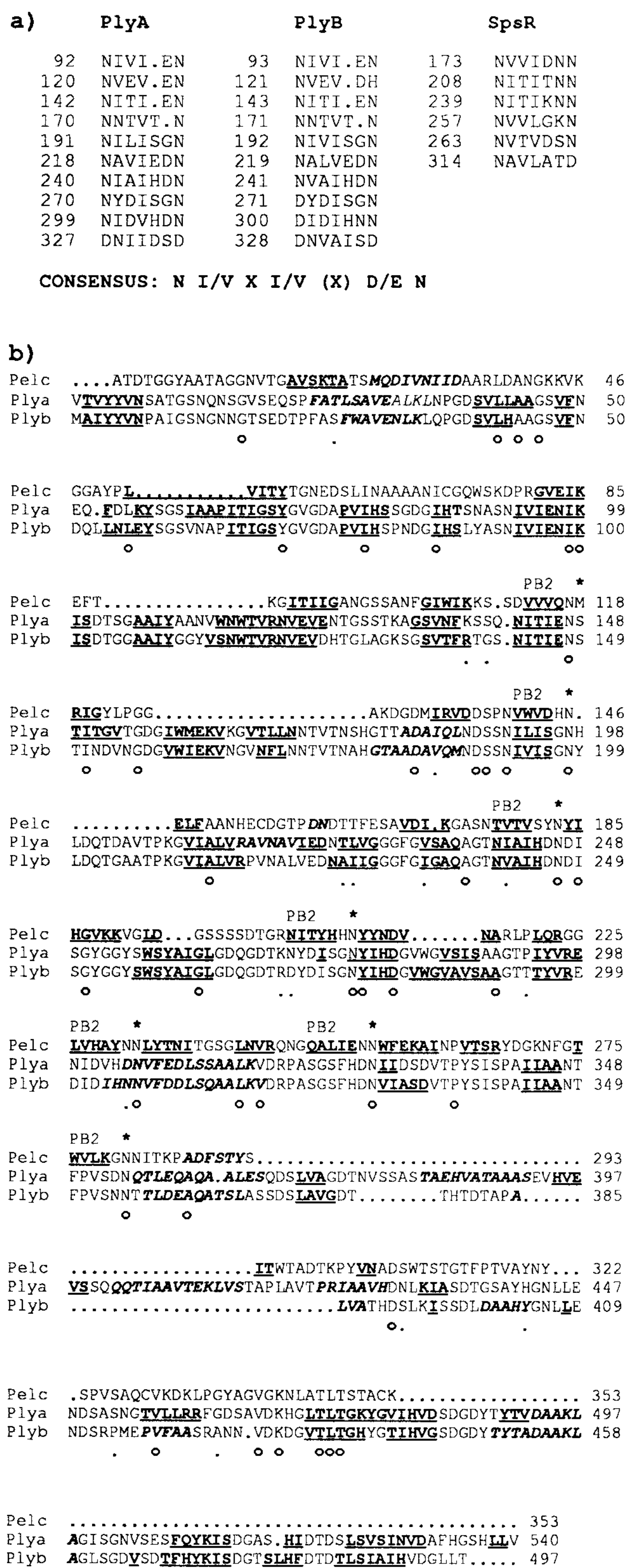

FIG. 5. (a) Repeat motifs found in PlyA, PlyB, and SpsR. The position in the sequence of the first amino acid in each repeat is indicated. A rough consensus sequence is shown. PlyA and PlyB each contain 10 copies of the motif; SpsR contains 6 copies. (b) Sequence alignment of PelC from *E. chrysanthemi* with PlyA and PlyB from *R. leguminosarum* bv. viciae, showing conservation of secondary structure. The mature form of PelC (lacking the N-terminal signal peptide) was used for the alignment. Residues that are conserved in all three proteins (○) and between PelC and either PlyA or PlyB (·) are indicated beneath the alignment. Structural elements are indicated in boldface type, with α-helices in italics and β-strands underlined. The secondary structure of PelC is known from the crystal structure (49). The β-strands that form the PB2 β-sheet in PelC are indicated. The residues which form stacks stabilizing the parallel β-helix of PelC are indicated by asterisks above the sequence. The stacks are composed mainly of Asn residues, which occur as the second residue after the end of the PB2 β-strands. The secondary structure of PlyA and PlyB was predicted by PHDsec (34) and aligns well with the known structure of PelC, suggesting that these proteins may have a similar fold.

the truncated form of Egl may indicate that this protein does not remain cell associated and might suggest a role for the N-terminal part of the protein in surface association. It is possible that the activities encoded by *plyA* and *plyB* are also predominantly cell associated, since the areas of clearing formed by *R. leguminosarum* bv. viciae on CMC and EPS agar are visible only directly underneath the colonies. If a freely diffusible activity were present, more diffuse clearing around the colonies would be expected. The small amount of 65-kDa protein in culture supernatants that is thought to correspond to PlyB may be due to the release of a relatively low proportion of the PlyB protein from the cell surface.

It was previously proposed that at least one of the proteins secreted via the Prs system was required for symbiotic nitrogen fixation. The evidence presented here demonstrates that PlyA and PlyB are not required for nitrogen fixation and that they do not have a strong effect on nodulation. Work with *R. meliloti* has demonstrated that a particular size range of EPS produced by the bacterium is required for activity in nodulation (2, 43). So far, two enzymes, encoded by *exoK* and *exsH*, that cleave the bacterial EPS have been identified in *R. meliloti* (5, 52). ExoK is thought to be secreted in a *sec*-dependent manner since it has a potential N-terminal transit peptide, and ExsH (which contains four RTX repeats) is thought to be a glycosyl hydrolase that is secreted via the *R. meliloti* PrsDE type I secretion system (52). These glycosyl hydrolases are not homologous to PlyA and PlyB.

The residual low level of degradation of CMC and EPS by the *plyA plyB* double mutant suggests that an additional polysaccharidase may be produced by *R. leguminosarum* bv. viciae. To demonstrate a requirement for these enzymes in the symbiosis, it may therefore be necessary to mutate additional genes. Although *plyA* mutants are apparently unaffected in the ability to degrade CMC and EPS, we have demonstrated, by placing *plyA* under the control of a constitutive promoter, that PlyA has glycanase activity. Our results suggest that under free-living conditions, *plyA* is not highly expressed, and they imply that *plyA* and *plyB* may be under different regulatory controls. An in situ analysis (with appropriate gene fusions) of *plyA* and *plyB* gene expression during legume nodulation would reveal if either gene is expressed during legume infection.

Four gene products have been identified so far that require *prs* genes for secretion from *R. leguminosarum* bv. viciae: NodO, PlyA, PlyB, and Egl (from *A. caulinodans*). Analysis of extracellular proteins indicates that other proteins are also secreted via the same system. SpsR from *Sphingomonas* may also be secreted via a type I secretion system. Like PlyA, it is encoded in a cluster of EPS-biosynthetic genes and is expected to be a secreted protein involved in EPS processing. SpsR has no N-terminal signal sequence (49) but does have a negatively charged residue followed by several hydrophobic residues at its extreme C terminus, a motif often present in proteins secreted via type I secretion systems (18). Two genes, *atrB* and *atrD*, encoding a type I secretion system are also present within the

sps gene cluster (49), and these genes may be required for the secretion of SpsR.

PlyA, PlyB, and SpsR share a novel repeat motif. These repeats may be involved in the formation of a similar structure to the parallel β-helix formed by the pectate lyase superfamily, on the basis of conservation of significant structural features. The repeat sequences align with the PB2 β-strands of PelC (50), and the terminal asparagines in the repeats align with the residues in PelC that form the asparagine ladder stabilizing a tight turn between PB2 and PB3. The conservation of these asparagines is particularly significant in view of the otherwise low overall sequence similarity. These features suggest the existence of a family of bacterial proteins involved in polysaccharide processing, including PlyA, PlyB, and SpsR, that are secreted by type I secretion systems.

The Egl protein shows no similarity to PlyA, PlyB, or any of the RTX family of proteins. Therefore, Egl lacks the RTX repeats, and we found no evidence for the PlyA-type heptapeptide repeat. However, Egl does contain several copies of a large (150-amino-acid) repeat that is similar to Ca^{2+}-binding domains of some proteins (17). Thus, Egl may be a member of a different class of polysaccharide degrading enzymes that are secreted via a type I system. It remains to be determined if the repeat domains in PlyA and PlyB or Egl play any role in their secretion. However, the observation that there are three classes of secreted proteins, each with a different type of internal-repeat structure, suggests that repeated domains may be important for the secretion or folding of these proteins in the extracellular environment.

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