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Cloning of an *Azorhizobium caulinodans* Endoglucanase Gene and Analysis of Its Role in Symbiosis

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***Azorhizobium caulinodans* ORS571, a symbiont of the tropical leguminous plant *Sesbania rostrata*, showed low, constitutive levels of endoglucanase (Egl) activity. A clone carrying the gene responsible for this phenotype was isolated via introduction of a genomic library into the wild-type strain and screening for transconjugants with enhanced Egl activity. By subcloning and expression in *Escherichia coli*, the Egl phenotype was allocated to a 3-kb *Eco*RI-*Bam*HI fragment. However, sequence analysis showed the *egl* gene to be much larger, consisting of an open reading frame of 1,836 amino acids. Within the deduced polypeptide, three kinds of putative domains were identified: a catalytic domain, two cellulose-binding domains, and an eightfold reiterated motif. The catalytic domain belongs to the family A of cellulases. A C-terminal stretch of 100 amino acids was similar to family II cellulose-binding domains. A second copy of this domain occurred near the middle of the polypeptide, flanked by reiterated motifs. ORS571 mutants carrying a Tn*5* insertion in the *egl* gene had lost the Egl activity. These mutants as well as Egl-overproducing strains showed a normal nodulation behavior, indistinguishable from wild-type nodulation on *Sesbania rostrata* under laboratory conditions.**

During the establishment of the symbiosis between rhizobia and leguminous plant species, bacteria invade plant tissues via root hairs or intercellular penetration (crack entry) (for a review, see reference 37). In most interactions, infection threads that cross several cell layers to reach a newly formed nodule meristem, in which bacteria are internalized into plant cells, are formed. At several stages of nodule development, plant cell walls are crossed by the bacteria, and it has been hypothesized that enzymes of plant and/or bacterial origin could be involved in degradation of cell wall polymers to facilitate invasion (1, 7, 24, 45). For instance, upon bacterial colonization, cell wall hydrolysis has been observed by electron microscopy at the root hair tip (7), ahead of the tip of growing infection threads, at sites where the infection thread is going to pass from one cell into another (44), and in infection droplets where bacteria are located prior to their uptake into the plant cell (10). The observed zones of hydrolysis were local, suggesting that plant enzymes were activated in the neighborhood of the rhizobia or that bacterial enzymes were released in a very controlled way. Indications of hydrolysis of cell wall material by plant enzymes during nodule initiation have been obtained by the observation of effects caused by purified Nod factors (in the absence of bacteria) on host plant roots. The rhizobial Nod factors are lipochitooligosaccharides, responsible for many of the early phenomena of nodule induction. Application of purified Nod factors to roots of *Vicia faba* caused the formation of structures, related to infection threads and with signs of cell wall degradation (42). On the other hand, several *Rhizobium* strains show low constitutive levels of pectate-, cellulose-, or hemicellulose-degrading activity (25, 28–30, 45). However, the corresponding genes have not been cloned, and mutants have not been analyzed.

Here, we report on the cloning of an endoglucanase gene (*egl*) from *Azorhizobium caulinodans* ORS571, a gram-negative bacterium that nodulates the tropical legume *Sesbania rostrata*. Infection of this host takes place via crack entry followed by infection thread formation and internalization (15, 16). Nodules are induced on roots and at the sites of dormant root primordia located on the stem (32, 41). We have observed that in free-living culture, outside the host plant, *A. caulinodans* exhibits a weak Egl activity. We have cloned and sequenced the gene that is responsible for this phenotype, mutated it, and studied the effect of the mutation on root and stem nodulation.

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MATERIALS AND METHODS

Bacterial growth conditions and nodulation assay. The bacterial strains and plasmids used in this study are described in Table 1. Physical maps of the plasmids are illustrated in Fig. 2. ORS571 and derivatives were grown on YEB medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mM $MgSO_4$) or on minimal medium (18) with appropriate antibiotics (200 μg of carbenicillin per ml, 50 μg of spectinomycin per ml, 10 μg of tetracycline per ml, and 40 μg of gentamicin per ml). Nodulation genes were induced with 10 μM naringenin or one equivalent of *S. rostrata* seedling exudates. *Escherichia coli* and derivatives were grown on Luria-Bertani medium (40) supplemented with carbenicillin (100 μg/ml). Nodulation tests were performed as previously described, and nitrogen fixation was determined by the acetylene reduction assay (43).

Screening of β-glucosidase, cellobiohydrolase, endoglucanase, and polygalacturonase activities. For endoglucanase or polygalacturonase assays, bacterial colonies or spots of bacterial cultures were grown overnight on an appropriate agar medium, overlaid with potassium phosphate-buffered (pH 6.8) agarose (1%) containing 0.2% carboxymethyl cellulose (CMC) or 0.2% polygalacturonic acid and incubated for 2 to 4 days. CMC-containing plates were stained with Congo red (0.1%). After two to three washes with 1 M NaCl, the appearance of a clear halo indicated Egl activity (8). Polygalacturonic acid was precipitated with 1% cetyltrimethyl ammonium bromide or stained with 0.01% ruthenium red.

For testing Egl activity in *E. coli* derivatives, the cells were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Analysis of ostazin brilliant-red hydroxyethyl cellulose degradation was as described by Biely et al. (6). β-Glucosidase and cellobiohydrolase activities were assayed by using the chromophoric substrates 2-chloro-4-nitrophenyl-β-D-glucoside and 2-chloro-4-nitrophenyl-β-cellobioside according to the method of Claeyssens (11).

Well assay for Egl activity. Bacteria were grown in YEB or Luria-Bertani medium until mid-log phase. Cells were pelleted and washed with 1 volume of 50 mM citrate buffer (pH 5.5) or 50 mM potassium phosphate buffer (pH 6.8). Cell culture supernatants were assayed either directly or after a 10-fold concentration by vacuum desiccation; alternatively, proteins were precipitated with ammonium sulfate (70%). Total extracts were obtained by ultrasonication or disruption with a French pressure cell (20,000 lb/in^2). Protein extract (100 μl) was pipetted into 5-mm wells drilled into a 0.2% CMC-agarose layer. After overnight incubation, staining and washing were done as described above. Putative xylanase activity of cell extracts was assayed with the same method, but CMC was replaced by xylan (0.2%).

TABLE 1. Bacterial strains and plasmids

Strain and its plasmid(s)	Characteristic(s)	Source or reference
E. coli		
MC1061	*araD139* Δ(*ara leu*) Δ*lacX74 galU galK hsr hsm*$^+$ *strA*	9
pPH1JI	Broad-host-range vector incompatible with pGV910	38
A. caulinodans		
ORS571	Wild-type strain	15
ORS571-3	*nodD* Tn*5* insertion strain	19
ORS571-SHX	*egl* mutant strain carrying Tn*5-1*	This work
ORS571-SH6	*egl* mutant strain carrying Tn*5-2*	This work
pRGC1	pLAFR1 cosmid containing *egl*	This work
pGV910-Cl	pGV910 derivative containing a 4.3-kb *Eco*RI *egl* fragment	This work
pGV910-B3	pGV910 derivative containing an 11.7-kb *Bam*HI *egl* fragment	This work
pDG1	pUC19 derivative containing a 4.3-kb *Eco*RI *egl* fragment	This work
pDG2, pDG3, pDG4, and pDG5	Subclones of the 4.3-kb fragment in pUC19 (Fig. 2)	This work
pDG1′	Opposite orientation of insert as in pDG1	This work

Cloning, sequencing, and construction of mutants. DNA manipulations were performed as described by Sambrook et al. (39). The DNA sequence was determined from both strands of overlapping subclones of the *egl* locus. Where necessary, primers were designed to resolve ambiguities. Sequence data were assembled and analyzed, using the GCG package (version 7; Genetics Computer Group, Madison, Wis.). Tn*5* insertion mutagenesis was performed as described by De Bruijn and Lupski (12). Homologous recombinants were isolated by incompatibility curing, using the plasmid pPH1JI, as described by Ruvkun et al. (38).

Hybridization conditions. Southern blots were prehybridized in 6× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0)–5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin)–0.5% sodium dodecyl sulfate–1 μg of denatured salmon sperm DNA per ml at 56°C for 1 h. Hybridizations were performed under the same conditions for 14 h. Probes (see Fig. 2) were labeled with [α-^{32}P]dCTP.

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the EMBL and GenBank databases under accession number Z48958.

RESULTS

Screening for polysaccharide-degrading activities in strain ORS571. Polygalacturonase and endoglucanase activities were screened by using the double-layer plate assay with polygalacturonic acid and CMC as the respective substrates (8). An Egl activity could be detected by this assay (Fig. 1), but polygalacturonase activity was not observed (data not shown). ORS571 cell extracts were tested for the presence of β-glucosidase and cellobiohydrolase activities, using chromophoric substrates (see Materials and Methods). Hydrolysis of the substrates was not observed, suggesting the absence of exoglucanases (data not shown). ORS571 cell extracts and culture supernatants were tested separately for Egl activity, using a well assay (see Materials and Methods). Egl activity was not detected in culture supernatants but was found in sonicated cell suspensions, indicating the cell-associated nature of the enzyme. Intact cells did not display Egl activity in this assay (Fig. 1).

The Egl activity was not influenced by incubation temperature (28 or 37°C), by growth in the presence of the substrate CMC, or by substrate degradation products such as glucose or cellobiose (data not shown). Naringenin, or plant root exudate, known to induce azorhizobial nodulation genes (18), had no effect on the Egl activity, and the *nodD* mutant strain ORS571-3 (19), lacking the nodulation gene activator NodD, produced the Egl activity at the same level as the wild-type strain (Fig. 1). Besides CMC, ostazin brilliant-red hydroxyethyl cellulose was also degraded, but xylan was not (data not shown).

Cloning of the ORS571 *egl* gene and expression in *E. coli*. To identify the gene responsible for the Egl activity, a genomic library of ORS571 DNA in the cosmid vector pLAFR1 (inserts

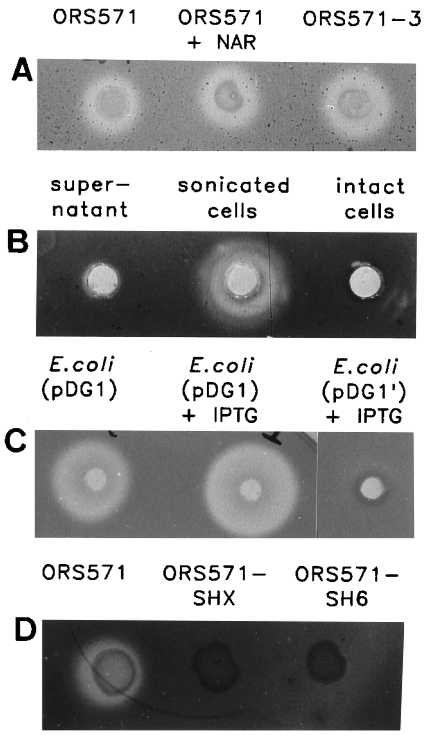

FIG. 1. Endoglucanase detection. Assays were performed with 0.2% CMC as the substrate. The presence of a cleared zone indicates enzyme activity. (A) Spot inoculations of cultures of ORS571, ORS571 grown with 10 μM naringenin (NAR), and the *nodD* mutant strain ORS571-3. (B) Location of the Egl activity. Samples were pipetted into 5-mm wells. (C) Expression of the 4.3-kb *Eco*RI fragment in *E. coli*. Plasmid pDG1′ contains the *egl* gene in the opposite direction with respect to the P*lac* promoter, compared with pDG1. (D) Egl activity from the wild type and from the *egl*::Tn*5* insertion mutants, ORS571-SH6 and ORS571-SHX.

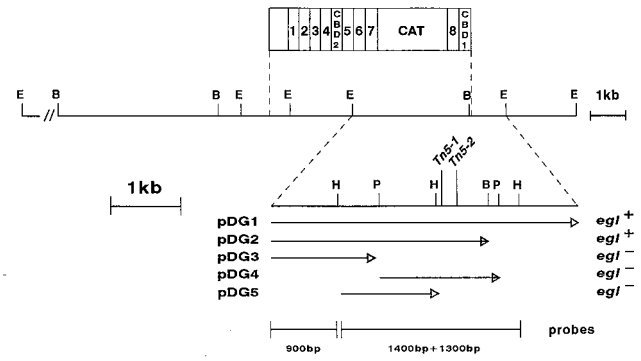

FIG. 2. Physical map of the *egl* locus and organization of the Egl polypeptide. Arrows show the direction of transcription from the P*lac* promoter of the vector pUC19. The ORF deduced from the nucleotide sequence analysis is schematically represented above the restriction map. Reiterated motifs correspond to the sequences rep1 to rep8 in Fig. 5. CAT, catalytic domain. The DNA fragments used as hybridization probes are indicated. B, *Bam*HI; E, *Eco*RI; H, *Hin*cII; P, *Pst*I.

of 20 to 30 kb) was introduced into *E. coli*. No Egl-positive colonies were found, presumably because of the lack of azorhizobial promoter function in *E. coli*. Therefore, the genomic library was introduced into ORS571 by triparental mating, and colonies with increased Egl activity were isolated. Four such colonies (of a total of 1,000 screened) contained an identical clone (pRGC1) with five *Eco*RI fragments. The Egl activity was ascribed to a 4.3-kb *Eco*RI DNA fragment by subcloning in pUC19 and expression analysis in *E. coli* (Fig. 2). The expression of the *egl* gene in *E. coli* relied on the *lacZ* promoter of pUC19. Indeed, Egl^+ *E. coli* derivatives all harbored the 4.3-kb fragment in the same orientation with respect to the *lacZ* promoter (reversion of the orientation caused loss of expression), and the Egl activity was influenced by IPTG and glucose (Fig. 1). Further subcloning of the 4.3-kb *Eco*RI fragment revealed that a 3-kb *Eco*RI-*Bam*HI fragment contained the information needed to confer the Egl phenotype to *E. coli* (pDG2; Fig. 2).

Sequence analysis of *egl*. Initially, the nucleotide sequence of the 3-kb *Eco*RI-*Bam*HI fragment of clone pDG2 was determined. One open reading frame (ORF) spanned the entire 3-kb fragment. When grown in the presence of IPTG and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), *E. coli*(pDG2) formed blue colonies, indicative of a translational fusion between the *egl* ORF and the α peptide from pUC19. Extension of the nucleotide sequence revealed that the *egl* ORF was 2,458 bp longer than pDG2 at the N terminus and 25 bp longer at the C terminus (Fig. 3). Thus, the N-terminal and C-terminal parts of the Egl protein were not essential for catalytic activity. A putative start codon was located at position 301 of the sequenced region (Fig. 3). Upstream from this position, several translational stop codons were present in all reading frames. The proposed ORF encodes a polypeptide of 1,836 amino acids, with a predicted molecular mass of 188,213 Da. Computer-assisted comparison of Egl with sequences from the GenBank and EMBL databases revealed three types of domains. Their overall organization is illustrated in Fig. 2. One domain (CAT; Fig. 2) showed similarity with the catalytic domain of endoglucanases belonging to the A family (23). At the C terminus, a stretch of 100 amino acids showed similarity with the family II cellulose-binding domains (CBDs) (4). The CBD is attached to the rest of the Egl polypeptide by a GPST-rich region that resembles a hinge region present in many multiple domain cellulases (17). A second CBD is located near the center of the Egl polypeptide, flanked by reiterated motifs, which constitute the third type of domain (Fig. 4). The reiterated motifs are present in eight copies (Fig. 2). Each copy is approximately 115 amino acids long and contains sequences that resemble hinge regions at their extremities. The reiterated motifs showed significant similarity with part of the Na^+-Ca^{2+} exchangers from mammals (Fig. 5) (33). The first 319 amino acids of the Egl polypeptide showed no significant similarity with any protein from the GenBank and EMBL databases and did not contain a signal sequence for protein export (46).

Hybridization analysis. To determine whether the ORS571 genome harbors *egl*-related sequences, a Southern blot hybridization was performed. *Eco*RI-digested total DNA was probed with a combination of two *Hin*cII fragments (1,300 and 1,400 bp; Fig. 2) corresponding to the catalytic domain, the C-terminal CBD, and two reiterated motifs. At low stringency, multiple *Eco*RI fragments hybridized (Fig. 6); one of them was the 1.7-kb *Eco*RI fragment present in pRGC1 (Fig. 2) with reiterated motifs similar to those present in the probe. The other DNA fragments that hybridized did not occur in pRGC1 and represented *egl*-related sequences present in the genome. Hybridization with a 900-bp *Hin*cII probe (Fig. 2), corresponding to reiterated motifs, revealed similarity only with the 1.7-kb *Eco*RI fragment. Therefore, sequences located outside the reiterated regions accounted for the hybridization of the 3.7-, 2.2-, 2.1-, and 1.8-kb *Eco*RI fragments with the large *Hin*cII probe.

The role of *egl* in nodulation. Mutations in the *egl* gene were generated by Tn*5* mutagenesis of the cosmid pRGC1 in *E. coli*. Plasmids harboring Tn*5* insertions were conjugated into strain ORS571. Transconjugants were screened for the loss of the Egl overexpression phenotype: two colonies that had reacquired a wild-type Egl phenotype were retained. The Tn*5* insertions were mapped within the 3-kb *Eco*RI-*Bam*HI fragment responsible for Egl activity in *E. coli* (Fig. 2). The Tn*5* insertions were homogenized in the ORS571 genome (see Materials and Methods). The mutant strains were completely Egl^- (Fig. 1). Egl activity was restored upon introduction of the cosmid pRGC1 or the subclone pGV910-C1. The latter plasmid harbors the 4.3-kb *Eco*RI fragment (Fig. 2 and Table 1). Therefore, also in *A. caulinodans*, expression of the catalytic domain seems to be sufficient for Egl activity. The expression in pGV910-C1 is presumably driven by the promoter of the chloramphenicol resistance gene from the vector (43).

The Egl^- mutants were inoculated on stems and roots of *S. rostrata*, and the nodulation capacity and the nitrogen-fixing ability were similar to those of the wild type (data not shown). Roots were inoculated with dilution series (10^8, 10^6, and 10^4 bacteria per plant) of mutant and wild-type cultures to detect eventual differences in nodulation efficiency. In addition, Egl-overproducing strains, ORS571(pGV910-B3) and ORS571(pGV910-C1), were inoculated on stems and roots of *S. rostrata*. The nodulation process was affected neither by the absence nor by the excess of the endoglucanase.

DISCUSSION

Evidence for the involvement of plant cell wall-degrading enzymes in the *Rhizobium*-legume symbiosis is mainly based on microscopical observations. The purpose of this study was to investigate the putative symbiotic role of a CMC-degrading endoglucanase present in *A. caulinodans*. The observed Egl activity was constitutive and cell associated.

An attempt to isolate the gene by its expression in *E. coli*

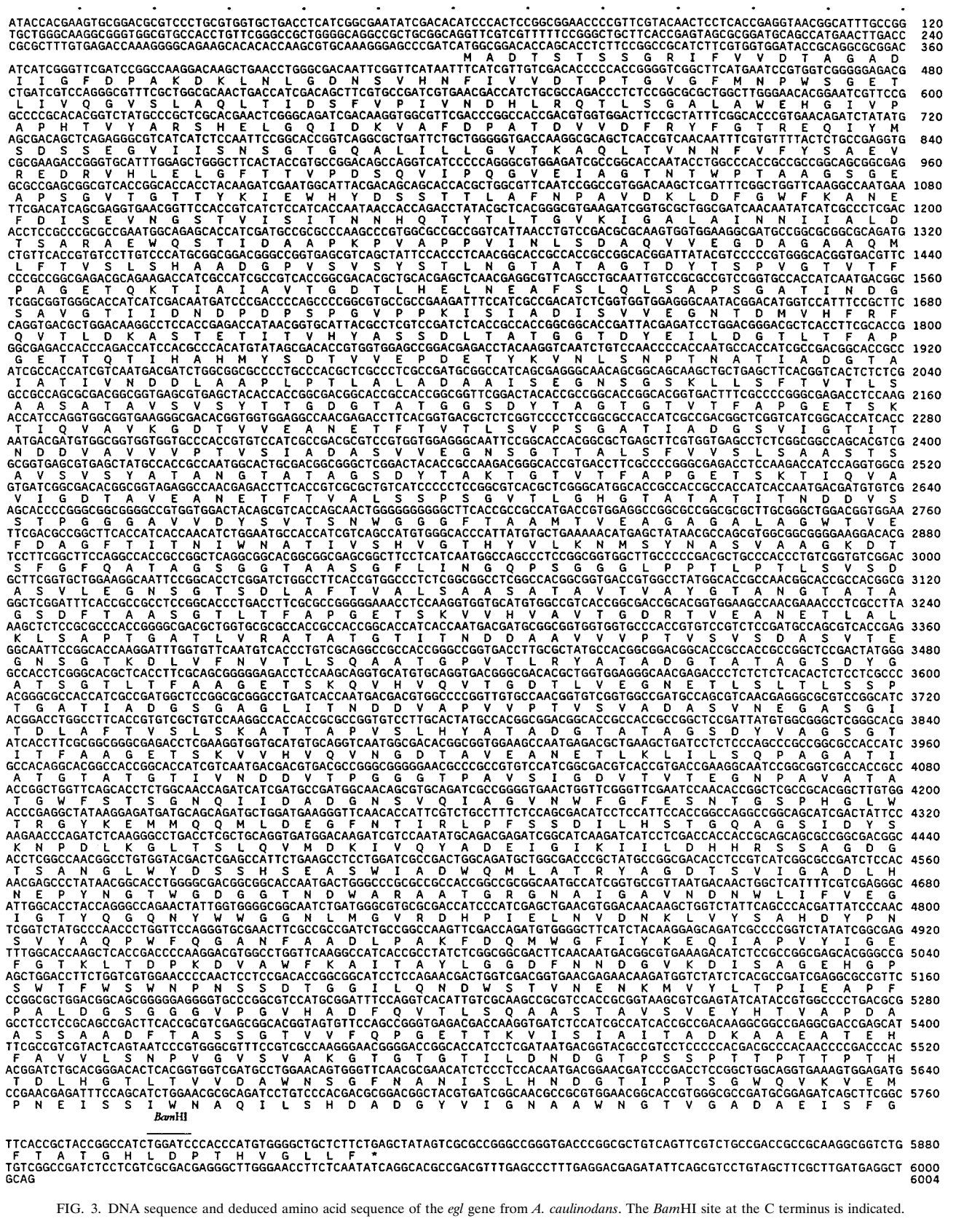

FIG. 3. DNA sequence and deduced amino acid sequence of the *egl* gene from *A. caulinodans*. The *Bam*HI site at the C terminus is indicated.

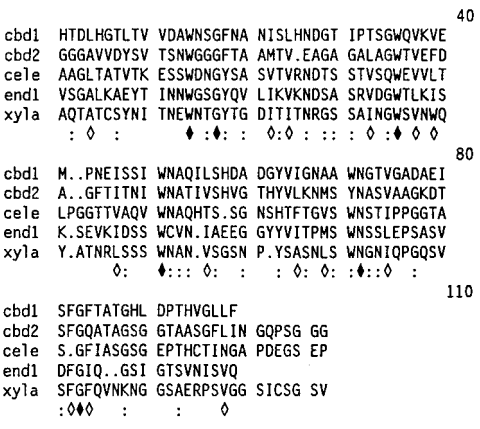

FIG. 4. Alignment of bacterial CBDs. cbd1 and cbd2, *A. caulinodans*; cele, *Thermomonospora fusca* (27); end1, *Butyrivibrio fibrisolvens* (5); xyla, *Pseudomonas fluorescens* (22). ◆, fully conserved residues; ◇, hydrophobic consensus (4 of 5 residues are V, L, I, M, A, or F); :, similar residues (4 of 5 are members of the same group: [A, G, S, T], [Q, D, E, N], [W, Y, F], [H, R, K]).

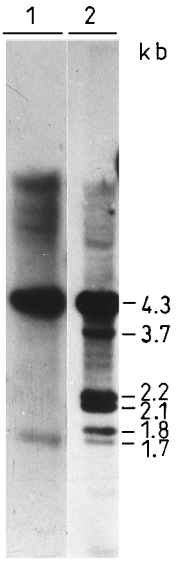

FIG. 6. Southern hybridization. Total ORS571 DNA was digested with *Eco*RI and separated on a 0.8% agarose gel. Lane 1, hybridization with a 900-bp *Hin*cII probe (Fig. 2). Lane 2, hybridization with a 1,300 + 1,400-bp *Hin*cII probe (Fig. 2). Sizes of hybridizing bands are indicated in kilobases.

failed, probably because of the lack of azorhizobial promoter activity in *E. coli*. To circumvent this problem, an ORS571 population harboring an ORS571 cosmid library was screened for individuals with increased Egl activity. In this way, the structural gene responsible for the Egl activity was cloned. In principle, this strategy could have allowed the isolation of cosmid clones containing a gene(s) involved in the positive regulation of *egl* expression. Such clones were not found; perhaps the action of the postulated regulator would require a specific effector, absent in our assay. Subcloning into pUC19 and expression into *E. coli* assigned the Egl activity to a 3-kb DNA fragment. This fragment, which was sufficient to confer CMC degradation, did not correspond to the full *egl* gene. DNA sequence analysis showed the presence of a much larger ORF coding for a protein of 1,836 amino acids and containing three types of domains. A catalytic domain was related to family A hydrolases (Fig. 2) and more particularly to the endoglucanases from *Bacillus polymyxa* (2) and *Xanthomonas campestris* (21) (with 45 and 37% of amino acids at identical positions, respectively). Two CBDs were present; they had little reciprocal homology, but both belong to the family II of CBDs. Deletion of the end of the C-terminal CBD had no effect on the hydrolysis of soluble CMC. For several cellulases, it was shown that CBDs are not essential for hydrolysis of soluble cellulose but that they enhanced the breakdown of crystalline cellulose (13, 26, 31). We have not analyzed this yet, but we found that the enzyme was specifically bound by Avicel (Merck), a type of crystalline cellulose (unpublished results). Reiterated sequences constituted the third type of domain. The reiterated domain showed similarity with part of the Na^+-Ca^{2+} transporters of mammals. By analogy with the involvement of reiterated domains of cellulases and xylanases in complex formation (3, 40), it is possible that the reiterated domains bind other proteins. Perhaps they are involved in the attachment of the Egl to the bacterial surface. Although the endoglucanase is likely to be secreted, a signal sequence was not found in the N-terminal part.

Interruption of the *egl* gene abolished all detectable endoglucanase activity. The mutant strains were not affected in their symbiotic behavior. Likewise, overexpression of the *egl* gene did not alter the nodulation behavior. Yet, a role in symbiosis cannot be excluded. Plant-derived hydrolases might be produced in sufficient amounts to overcome a necessity for the bacterial endoglucanase. In addition, ORS571 could carry a cryptic *egl* gene(s) that is not expressed under the conditions

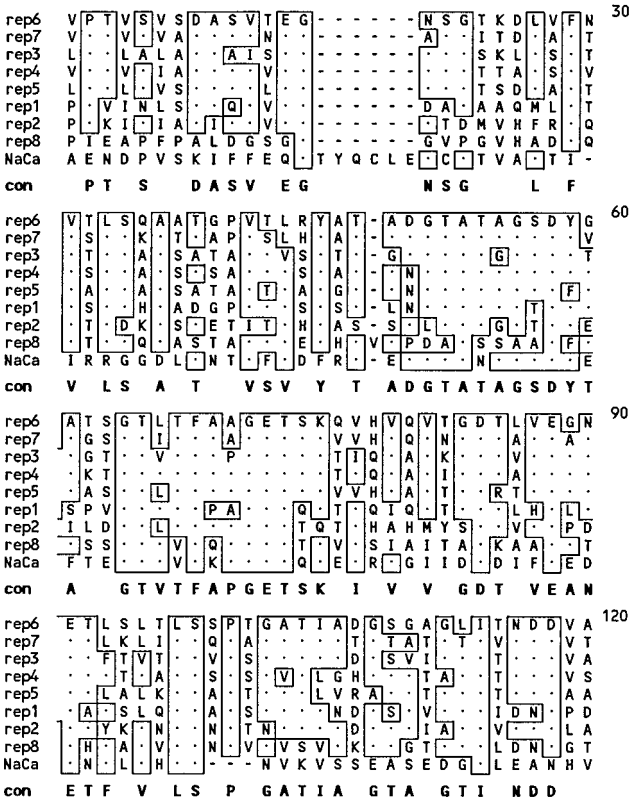

FIG. 5. Alignment of the eightfold reiterated motifs (rep) from *A. caulinodans* Egl with part of the intracellular loop of cardiac sarcolemmal Na^+-Ca^{2+} exchanger (NaCa) (33). The digits indicate the positions of the domains presented in Fig. 2. Dots indicate identical amino acids; con, consensus (at least 5 of 9 residues identical). Dashes indicate gaps for optimal alignment.

tested here. Indications supporting this assumption came from hybridization experiments. A putative symbiotic function of the Egl protein, not investigated in this study, is the liberation of bacteria from senescent nodules. *S. rostrata* forms determinate nodules with a limited lifetime (32). It could be an advantage for the bacteria to leave the plant cells when the nodules are senescent. The escape of bacteroids from nodule tissue is perhaps facilitated by the action of endoglucanases.

In general, cellulolytic organisms produce cellulases to break down cellulose to cellobiose and glucose, which are used as carbon sources. Because *A. caulinodans* cannot grow on glucose or cellobiose (14), it is very unlikely that the Egl enzyme would serve this purpose. Cellulases might also assist in the release of host plant nutrients, as suggested for *X. campestris* (20), *Erwinia carotovora* (47), and *Pseudomonas solanacearum* (36). We have no evidence that this would be the case for ORS571. An example of an endoglucanase-producing bacterium that does not feed on cellulose is the genus *Azoarcus* (34, 35). This bacterium was found to infect the roots of Kallar grass, a process perhaps facilitated by the production of cellulolytic enzymes.

Because of the fact that cryptic genes can complement a knockout mutation, the search for a biological role for bacterial endoglucanases is made difficult. A study of the substrate specificity of the cloned enzyme, on the one hand, and the use of expression libraries to identify other, related *Azorhizobium* functions by their expression in *E. coli*, on the other hand, may lead to a better understanding of the role of cellulolytic enzymes in the life of bacteria that do not grow on the breakdown products.

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REFERENCES

1. **Al-Mallah, M. K., M. R. Davey, and E. C. Cocking.** 1987. Enzymatic treatment of clover root hairs removes a barrier to Rhizobium-host specificity. Bio/Technology **5:**1319–1322.
2. **Baird, S. D., D. A. Johnson, and V. L. Seligy.** 1990. Molecular cloning, expression, and characterization of endo-β-1,4-glucanase genes from *Bacillus polymyxa* and *Bacillus circulans*. J. Bacteriol. **172:**1576–1586.
3. **Bayer, E. A., E. Morag, and R. Lamed.** 1994. The cellulosome—a treasure-trove for biotechnology. Trends Biotechnol. **12:**379–386.
4. **Béguin, P., and J.-P. Aubert.** 1994. The biological degradation of cellulose. FEMS Microbiol. Rev. **13:**25–58.
5. **Berger, E., W. A. Jones, D. T. Jones, and D. R. Woods.** 1989. Cloning and sequencing of an endoglucanase (*end1*) gene from *Butyrivibrio fibrisolvens* H17c. Mol. Gen. Genet. **219:**193–198.
6. **Biely, P., D. Mislovičová, and R. Toman.** 1985. Soluble chromogenic substrates for the assay of endo-1,4-β-xylanases and endo-1,4-β-glucanases. Anal. Biochem. **144:**142–146.
7. **Callaham, D. A., and J. G. Torrey.** 1981. The structural basis for infection of root hairs of *Trifolium repens* by *Rhizobium*. Can. J. Bot. **59:**1647–1664.
8. **Carder, J. H.** 1986. Detection and quantitation of cellulase by Congo red staining of substrates in a cup-plate diffusion assay. Anal. Biochem. **153:**75–79.
9. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179–207.
10. **Chalifour, F.-P., and N. Benhamou.** 1989. Indirect evidence for cellulase production by *Rhizobium* in pea root nodules during bacteroid differentiation: cytochemical aspects of cellulose breakdown in rhizobial droplets. Can. J. Microbiol. **35:**821–829.
11. **Claeyssens, M.** 1988. The use of chromophoric substrates and specific assays in the study of structure-activity relationships of cellulolytic enzymes, p. 393–397. *In* J.-P. Aubert, P. Béguin, and J. Millet (ed.), Biochemistry and genetics of cellulose degradation. Academic Press, London.
12. **De Bruijn, F. J., and J. R. Lupski.** 1984. The use of transposon Tn*5* mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids—a review. Gene **27:**131–149.
13. **Din, N., N. R. Gilkes, B. Tekant, R. C. Miller, Jr., R. A. J. Warren, and D. G. Kilburn.** 1991. Non-hydrolytic disruption of cellulose fibres by the binding domain of a bacterial cellulase. Bio/Technology **9:**1096–1099.
14. **Dreyfus, B.** Personal communication.
15. **Dreyfus, B., and Y. R. Dommergues.** 1981. Nitrogen-fixing nodules induced by *Rhizobium* on the stem of the tropical legume *Sesbania rostrata*. FEMS Microbiol. Lett. **10:**313–317.
16. **Duhoux, E.** 1984. Ontogénèse des nodules caulinaires du *Sesbania rostrata* (légumineuses). Can. J. Bot. **62:**982–994.
17. **Gilkes, N. R., B. Henrissat, D. G. Kilburn, R. C. Miller, Jr., and R. A. J. Warren.** 1991. Domains in microbial β-1,4-glycanases: sequence conservation, function, and enzyme families. Microbiol. Rev. **55:**303–315.
18. **Goethals, K., M. Gao, K. Tomekpe, M. Van Montagu, and M. Holsters.** 1989. Common *nodABC* genes in *Nod* locus 1 of *Azorhizobium caulinodans*: nucleotide sequence and plant-inducible expression. Mol. Gen. Genet. **219:**289–298.
19. **Goethals, K., G. Van den Eede, M. Van Montagu, and M. Holsters.** 1990. Identification and characterization of a functional *nodD* gene in *Azorhizobium caulinodans* strain ORS571. J. Bacteriol. **172:**2658–2666.
20. **Gough, C. L., J. M. Dow, C. E. Barber, and M. J. Daniels.** 1988. Cloning of two endoglucanase genes of *Xanthomonas campestris* pv. *campestris*: analysis of the role of the major endoglucanase in pathogenesis. Mol. Plant Microbe Interact. **1:**275–281.
21. **Gough, C. L., J. M. Dow, J. Keen, B. Henrissat, and M. J. Daniels.** 1990. Nucleotide sequence of the *engXCA* gene encoding the major endoglucanase of *Xanthomonas campestris* pv. *campestris*. Gene **89:**53–59.
22. **Hall, J., G. P. Hazlewoord, N. S. Huskisson, A. J. Durrant, and H. J. Gilbert.** 1989. Conserved serine-rich sequences in xylanase and cellulase from *Pseudomonas fluorescens* subspecies *cellulosa*: internal signal sequence and unusual protein processing. Mol. Microbiol. **3:**1211–1219.
23. **Henrissat, B., M. Claeyssens, P. Tomme, L. Lemesle, and J.-P. Mornon.** 1989. Cellulase families revealed by hydrophobic cluster analysis. Gene **81:**83–95.
24. **Higashi, S., and M. Abe.** 1980. Scanning electron microscopy of *Rhizobium trifolii* infection sites on root hairs of white clover. Appl. Environ. Microbiol. **40:**1094–1099.
25. **Hubbell, D. H., V. M. Morales, and M. Umali-Garcia.** 1978. Pectolytic enzymes in *Rhizobium*. Appl. Environ. Microbiol. **35:**210–213.
26. **Klyosov, A. A.** 1990. Trends in biochemistry and enzymology of cellulose degradation. Biochemistry **29:**10577–10585.
27. **Lao, G., G. S. Ghangas, E. D. Jung, and D. B. Wilson.** 1991. DNA sequences of three β-1,4-endoglucanase genes from *Thermomonospora fusca*. J. Bacteriol. **173:**3397–3407.
28. **Lopez, M., and E. Singer.** 1987. Degradative enzymes in *Rhizobium meliloti*, p. 185–187. *In* D. P. S. Verma and N. Brisson (ed.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
29. **Martinez-Molina, E., V. M. Morales, and D. H. Hubbell.** 1979. Hydrolytic enzyme production by *Rhizobium*. Appl. Environ. Microbiol. **38:**1186–1188.
30. **Mateos, P. F., J. I. Jimenez-Zurdo, J. Chen, A. S. Squartini, S. K. Haack, E. Martinez-Molina, D. H. Hubbell, and F. B. Dazzo.** 1992. Cell-associated pectinolytic and cellulolytic enzymes in *Rhizobium leguminosarum* biovar trifolii. Appl. Environ. Microbiol. **58:**1816–1822.
31. **Millward-Sadler, S. J., D. M. Poole, B. Henrissat, G. P. Hazlewood, J. H. Clarke, and H. J. Gilbert.** 1994. Evidence for a general role for high-affinity non-catalytic cellulose binding domains in microbial plant cell wall hydrolases. Mol. Microbiol. **11:**375–382.
32. **Ndoye, I., F. de Billy, J. Vasse, B. Dreyfus, and G. Truchet.** 1994. Root nodulation of *Sesbania rostrata*. J. Bacteriol. **176:**1060–1068.
33. **Nicoll, D. A., S. Longoni, and K. D. Philipson.** 1990. Molecular cloning and functional expression of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. Science **250:**562–565.
34. **Reinhold-Hurek, B., T. Hurek, M. Claeyssens, and M. Van Montagu.** 1993. Cloning, expression in *Escherichia coli*, and characterization of cellulolytic enzymes of *Azoarcus* sp., a root-invading diazotroph. J. Bacteriol. **175:**7056–7065.
35. **Reinhold-Hurek, B., T. Hurek, M. Gillis, B. Hoste, M. Vancanneyt, K. Kersters, and J. De Ley.** 1993. *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. Int. J. Syst. Bacteriol. **43:**574–584.
36. **Roberts, D. P., T. P. Denny, and M. A. Schell.** 1988. Cloning of *egl* gene of *Pseudomonas solanacearum* and analysis of its role in phytopathogenicity. J. Bacteriol. **170:**1445–1451.

37. **Rolfe, B. G., and P. M. Gresshoff.** 1988. Genetic analysis of legume nodule initiation. Annu. Rev. Plant Physiol. Plant Mol. Biol. **39:**297–319.
38. **Ruvkun, G. B., V. Sundaresan, and F. M. Ausubel.** 1982. Directed transposon Tn5 mutagenesis and complementation analysis of Rhizobium meliloti symbiotic nitrogen fixation genes. Cell **29:**551–559.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Tokatlidis, K., S. Salamitou, P. Béguin, P. Dhurjati, and J.-P. Aubert.** 1991. Interaction of the duplicated segment carried by *Clostridium thermocellum* cellulases with cellulosome components. FEBS Lett. **291:**185–188.
41. **Tsien, H. C., B. L. Dreyfus, and E. L. Schmidt.** 1983. Initial stages in the morphogenesis of nitrogen-fixing stem nodules of *Sesbania rostrata*. J. Bacteriol. **156:**888–897.
42. **van Brussel, A. A. N., R. Bakhuizen, P. C. van Spronsen, H. P. Spaink, T. Tak, B. J. J. Lugtenberg, and J. W. Kijne.** 1992. Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of *Rhizobium*. Science **257:**70–72.
43. **Van den Eede, G., R. Deblaere, K. Goethals, M. Van Montagu, and M. Holsters.** 1992. Broad host range and promoter selection vectors for bacteria that interact with plants. Mol. Plant Microbe Interact. **5:**228–234.
44. **van Spronsen, P. C., R. Bakhuizen, A. A. N. van Brussel, and J. W. Kijne.** 1994. Cell wall degradation during infection thread formation by the root nodule bacterium Rhizobium leguminosarum is a two-step process. Eur. J. Cell Biol. **64:**88–94.
45. **Verma, D. P. S., and V. Zogbi.** 1978. A cooperative action of plant and *Rhizobium* to dissolve the host cell wall during development of root nodule symbiosis. Plant Sci. Lett. **13:**137–142.
46. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14:**4683–4690.
47. **Walker, D. S., P. J. Reeves, and G. P. C. Salmond.** 1994. The major secreted cellulase, CelV, of *Erwinia carotovora* subsp. *carotovora* is an important soft rot virulence factor. Mol. Plant Microbe Interact. **7:**425–431.