

Rhamnogalacturonate Lyase RhiE Is Secreted by the Out System in *Erwinia chrysanthemi*

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Supernatants of rhamnose-induced *Erwinia chrysanthemi* strain 3937 cultures contain a principal secreted protein named RhiE. A *rhiE* mutant has been found among a set of rhamnose-induced MudI1681 *lacZ* fusions. RhiE is a 62-kDa protein that has rhamnogalacturonate lyase activity on rhamnogalacturonan I (RG-I). It does not require a divalent cation for its activity and has an optimal pH of 6.0. *rhiE* expression is strongly induced in the presence of rhamnose but is also regulated by PecT and Crp, two regulators of the transcription of pectinolytic enzyme genes. RhiE is secreted through the type II Out secretion pathway. RhiE has no disulfide bond. The absence of RhiE secretion in a *dsb* mutant indicated that disulfide bond formation is required for the biogenesis of the secretion apparatus. RhiE was searched for in several *E. chrysanthemi* strains by using antibodies, and it was found to be present in one-third of the strains tested. However, the reduced virulence of the *rhiE* mutant indicates that degradation of the RG-I region of pectin is important for full virulence of *E. chrysanthemi*.

Pectin is one of the main components present in plant cell walls. The pectin molecule can have three types of backbone: homogalacturonan, rhamnogalacturonan I (RG-I), and RG-II. Homogalacturonan (polygalacturonate [PGA]) is a polymer of galacturonic acid residues linked by α 1-4 bonds which are partially methylated and acetylated. Homogalacturonan forms the smooth regions of pectin which are interspersed with hairy regions of RG-I and RG-II. RG-I has a backbone composed of as many as 100 repeats of the disaccharide α -L-rhamnose- α -1,4-galacturonic acid. Side chains of arabinan or galactan are attached to O-4 of the rhamnosyl residues. RG-II is a complex polysaccharide with a short homogalacturonan backbone to which are linked side chains containing more than 10 different types of sugars (1).

Plant-pathogenic microorganisms often secrete enzymes that are able to degrade the polysaccharides of cell walls in order to disorganize the plant tissue and obtain carbon sources for their growth. The enzymes that degrade the smooth regions of pectin have been extensively studied (26). The action of pectin methylesterases and of pectin acetylerases liberates PGA. PGA is then degraded by pectate lyases or polygalacturonases that cleave either in an endo or in an exo mode. These enzymes have been characterized in bacteria as well in fungi. Much less is known about the enzymes degrading RG-I and RG-II. It seems that the presence of side chains prevents the degradation of the homogalacturonan backbone of RG-II by polygalacturonase and pectate lyases, and the debranching enzymes have not yet been characterized. Only a few enzymes that are able to cleave the RG-I backbone have so far been characterized, mostly in *Aspergillus aculeatus*. A rhamnogalac-

turonate lyase (18) cleaves inside the chain between a rhamnose and a galacturonate, creating an unsaturation on the galacturonate at the nonreducing end. A rhamnogalacturonate rhamnohydrolase (17) and a rhamnogalacturonate galacturonohydrolase (16) cleave the rhamnose and galacturonate residues, respectively, present at the nonreducing end of the rhamnogalacturonate chain. Other enzymes with putative rhamnogalacturonase activity have been described for *Trametes sanguinea* (28) and *Neurospora crassa* (20). Up to now, only one bacterial enzyme that is able to cleave RG-I has been described: the rhamnogalacturonate lyase of *Pseudomonas cellulosa* (14).

Erwinia chrysanthemi is an enterobacterium that provokes the soft rot disease of many plant species. It secretes, in the outer medium, enzymes that degrade the components of the cell walls: pectinases, cellulases, and proteases. At least eight pectinases and the cellulase Cel5 are secreted by the type II Out secretion system, while four proteases are secreted by a type I secretion machinery (26). A type III secretion system allows for secretion of the harpin HrpN (2). *E. chrysanthemi* possesses a battery of enzymes that allow it to degrade the PGA region of pectin and to then use it as the sole carbon source for its growth. A pectin acetylerase (PaeY), two pectin methylesterases (PemA and PemB), eight endo-pectate lyases (PelA, PelB, PelC, PelD, PelE, PelI, PelL, and PelZ), two exo-pectate lyases (PelW and PelX), and three polygalacturonases (PehV, PehW, and PehX) have been characterized (26). The oligogalacturonates produced are then metabolized and enter the bacterial general metabolism. Synthesis of all of these enzymes is finely controlled by a set of transcriptional regulators (KdgR, PecS, PecT, cyclic AMP receptor protein [CRP], and ExpR) that respond to environmental signals (9, 32). Thus, the *E. chrysanthemi* PGA pathway is very elaborate and allows complete degradation of the smooth region of pectin. It would seem unlikely that *E. chrysanthemi* could degrade the smooth regions of pectin to completion but leave the RG-I

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regions intact. The synthesis of enzymes involved in PGA degradation is induced in the presence of galacturonate. To identify the enzymes involved in RG-I degradation, we looked for rhamnose-inducible secreted proteins. We characterized the gene *rhiE*, encoding a secreted rhamnogalacturonate lyase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. *E. chrysanthemi* and *Escherichia coli* cells were grown at 30 and 37°C, respectively, in Luria broth (LB) medium, M63 minimal medium, or MacConkey medium (15) supplemented with a carbon source (0.2%) and, when required, with antibiotics at the following concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; and chloramphenicol, 20 µg/ml.

Genetic techniques. Transduction with phage φEC2 was carried out as described by Résibois et al. (23). Marker exchange recombinations were obtained after growth in low-phosphate medium, as described by Roeder and Collmer (27). Mutagenesis was performed with miniMu MudI1681 (6). A 0.2-ml portion of the lysate of phage MudI1681 was added to 0.2 ml of an overnight culture of strain A350. After 20 min, the bacteria were spread onto LB-kanamycin agar plates. Colonies formed after 24 h were replica plated onto MacConkey-lactose and MacConkey-lactose-rhamnose agar plates.

Recombinant DNA techniques. Preparations of chromosome and plasmid DNAs, restriction digestions, ligations, DNA electrophoresis, transformations, and electroporations were carried out as described by Sambrook et al. (30). Sequencing was performed by Genome Express SA (Grenoble, France). Site-directed mutagenesis was performed with the QuickChange kit (Stratagene).

SDS-PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (13). After electrophoresis, the proteins were electroblotted onto nitrocellulose in a semidry apparatus at 2mA/cm² for 20 min in transfer buffer containing 40 mM glycine, 50 mM Tris, 0.4% SDS, and 10% methanol. The nitrocellulose was then saturated with gelatin, incubated with antibodies, and developed with the ECL enhanced chemiluminescence detection kit (Pharmacia-Amersham Biotech). Anti-RhiE antibodies were diluted 1:2,000.

Preparation of RhiE antibodies. Strain A350 was grown overnight in LB medium containing rhamnose. After centrifugation, the proteins contained in the supernatants were precipitated in 50% ethanol and loaded onto preparative SDS-10% polyacrylamide gels. The band containing RhiE was cut out, and the protein was electroeluted. The protein was injected into a rabbit for antibody production by Valbex (Villeurbanne, France).

Protein labeling. Overexpression and exclusive labeling of plasmid-encoded proteins were carried out with the T7 promoter-T7 polymerase system of Tabor and Richardson (33).

Pathogenicity test. Pathogenicity tests were performed on chicory leaves. Ten microliters of M63 medium-grown bacteria diluted to an optical density at 600 nm of 0.05 were inoculated into a leaf wounded with a scalpel. Leaves were maintained for 40 h in a dew chamber, and the length of rotted tissue was measured.

Enzyme assays. β-Glucuronidase assays were performed with toluenized cells grown to exponential phase with *p*-nitrophenyl-β-D-glucuronate as the substrate (21). β-Galactosidase assays were performed with toluenized cells grown to exponential phase with *o*-nitrophenyl-β-D-galactose as the substrate (15). Rhamnogalacturonate lyase activity was monitored by measuring the increase of absorbance at 230 nm in a reaction medium containing 0.05% RG-I and enzymatic extract in 100 mM Tris-HCl or citrate-phosphate buffer at 37°C. RG-I was prepared as described by Bonnin et al. (3). Briefly, sugar beet pectin was dispersed in 0.1 M NaOH at 90°C. After 2 h, the pH was adjusted to 6 with HCl and the extracted polymers were precipitated with 3 volumes of ethanol. The precipitate was hydrolyzed with 0.5 M HCl at 80°C. After dialysis and freeze-drying, rhamnogalacturonan (rhamnose/galactose/galacturonic acid molar ratio of 1:1.2:2.8) was recovered.

Nucleotide sequence accession number. The complete DNA sequence of *rhiE* will appear in nucleotide databases under accession number AJ438339.

RESULTS

***E. chrysanthemi* secretes a rhamnose-inducible protein.** While galacturonate is metabolized by *E. chrysanthemi*, rhamnose, the other main component of pectin, is not. None of the

20 *E. chrysanthemi* strains tested was able to use rhamnose as the sole carbon source for its growth (Table 2). In addition, no mutant able to grow on agar plates containing rhamnose as the sole carbon source was observed even after several days, in contrast to observations with lactose, 2-keto-3-deoxygluconate, or glucuronate, for which the degradation pathway exists but is not expressed in the wild-type strain (8, 11, 12). This suggests that *E. chrysanthemi* does not possess a complete rhamnose degradation pathway. However, when the proteins present in the supernatant of a culture of strain A350 grown in the absence or presence of rhamnose were analyzed by SDS-PAGE, a 62-kDa rhamnose-induced protein could be observed, indicating that rhamnose can enter the bacteria and turn on the expression of some genes.

To find the gene of this secreted protein, we performed mutagenesis of strain A350 with phage MudI1681, which allows for the formation of a transcriptional fusion with the *lacZ* reporter gene. We looked for colonies which were white on MacConkey-lactose plates and pink or red on MacConkey-lactose-rhamnose plates. Ten mutants containing fusions in *rhi* (rhamnose-inducible) genes were found. The supernatants of cultures of these 10 mutants grown in LB-rhamnose medium were analyzed by SDS-PAGE. In one of them, A3749, the 62-kDa rhamnose inducible protein was absent. The mutated gene was named *rhiE*.

Cloning and sequence of *rhiE*. To clone the wild-type allele of *rhiE*, we first cloned the region upstream of the point of insertion of MudI1681. The *rhiE*::MudI1681 allele was cloned in vivo on an R110 plasmid. The *R*'*rhiE* plasmid was digested with *Bam*HI, and the fragments were ligated in pBluescript. Plasmids containing the *lacZ* gene and the adjacent *E. chrysanthemi* DNA were identified as blue colonies after the transformation of *E. coli* MC1061 and selection on LB-ampicillin-X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates. Analysis of the plasmid content of Lac⁺ colonies obtained by *Bam*HI digestion showed that their insert contained, besides the *lacZ* gene, 1.7 kb of *E. chrysanthemi* DNA (Fig. 1). Establishment of a restriction map of this DNA fragment showed that it contained a unique *Eco*RI site. A Cm^r cassette was inserted into this site, and this construction was then introduced into *E. chrysanthemi* and recombined into the chromosome. The resulting strain, A3884, did not produce RhiE, indicating that the *Eco*RI site is within the *rhiE* gene. DNA of strain A3884 was extracted, digested with *Bgl*II or *Nsi*I (two enzymes that do not cut the Cm^r cassette), and ligated with pBluescript digested with *Bam*HI or *Pst*I. After the transformation of *E. coli*, Cm^r clones were selected. The plasmids obtained with *Bgl*II contained the largest inserts, with more than 6 kb of chromosomal DNA (Fig. 1). A partial digestion with *Eco*RI allowed for the excision of the Cm^r cassette of the plasmid and restored the wild-type allele of *rhiE*. A 2,420-bp DNA fragment surrounding the *Eco*RI site was sequenced. It contained a single open reading frame beginning at nucleotide 369 and ending at nucleotide 2159 and was followed by a sequence that could act as a Rho-independent terminator. However, the putative GTG initiation codon was not preceded by a classical Shine-Dalgarno sequence (Fig. 2). To check whether this GTG was the initiation codon, it was modified by site-directed mutagenesis to GTA. Synthesis of RhiE, checked by exclusive labeling of the product of the gene cloned under

TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant characteristics	Reference or source
Bacterial strains		
<i>E. chrysanthemi</i> 3937 derivatives		
A350	<i>lmr</i> ^{T^c} <i>lacZ</i>	11
A837	<i>lmr</i> ^{T^c} <i>lacZ kdgR</i>	8
A1524	<i>lmr</i> ^{T^c} <i>lacZ pecS::Mu-Cm^r</i>	25
A2174	<i>lmr</i> ^{T^c} <i>lacZ pecT::Cm^r</i>	7
A2507	<i>lmr</i> ^{T^c} <i>lacZ crp::Cm^r</i>	24
A2757	<i>lmr</i> ^{T^c} <i>lacZ expl::Cm^r</i>	19
A2107	<i>lmr</i> ^{T^c} <i>lacZ dsbA::uidA-kan dsbC-Cm^r</i>	Laboratory collection
A3657	<i>lmr</i> ^{T^c} <i>lacZ pecS::Mu-Cm^r ΔoutD</i>	V. E. Shevchik
A3749	<i>lmr</i> ^{T^c} <i>lacZ rhiE::MudI1681</i>	This work
A3884	<i>lmr</i> ^{T^c} <i>lacZ rhiE::Cm^r</i>	This work
A3946	<i>lmr</i> ^{T^c} <i>lacZ rhiE::uidA-Km</i>	This work
A3981	<i>lmr</i> ^{T^c} <i>lacZ kdgR::Tn5-B21 ΔoutC</i>	V. E. Shevchik
A3982	<i>lmr</i> ^{T^c} <i>lacZ kdgR::Tn5-B21 ΔPDZoutC</i>	V. E. Shevchik
Other <i>E. chrysanthemi</i> strains		
EC16		Laboratory collection
ENA49		Laboratory collection
ET3		E. Lojkowska
4073		E. Lojkowska
1240 (ICPB EC174)		E. Lojkowska
SF109-1		E. Lojkowska
4072		E. Lojkowska
1271 (NCPBP 1065)		E. Lojkowska
4040		E. Lojkowska
722		E. Lojkowska
1270 (NCPBP)		E. Lojkowska
4062		E. Lojkowska
SH230-C143		E. Lojkowska
1342 (ICPB EC239)		E. Lojkowska
1891		E. Lojkowska
4060		E. Lojkowska
4061		E. Lojkowska
4065		E. Lojkowska
3367		E. Lojkowska
3716		E. Lojkowska
CIP366		E. Lojkowska
<i>E. carotovora</i> subsp. <i>odorifera</i> 56–487		Laboratory collection
<i>E. carotovora</i> subsp. <i>betavasculorum</i> 52–479		Laboratory collection
<i>E. carotovora</i> subsp. <i>carotovora</i> SCRI193		Laboratory collection
<i>E. carotovora</i> subsp. <i>atroseptica</i> SCRI31		Laboratory collection
<i>E. rhapontici</i> 49–421		Laboratory collection
<i>E. cypripedi</i> 51–440		Laboratory collection
<i>E. carnegiana</i> 55–483		Laboratory collection
<i>E. coli</i>		
NM522	Δ(<i>lac-proAB</i>) <i>thi hsd-5 supE</i> (F' <i>proAB</i> ⁺ <i>lacI</i> ^q ΔM15)	Stratagene
MC1061	F ⁻ <i>araD139 Δ(ara-leu)7696 galE15 galK16 ΔlacX74 rpsL hsdR2</i> (r _K ⁻ m _K ⁻) <i>mcrA mcrB1</i>	Laboratory collection
BL21(DE3)	<i>E. coli</i> B; F ⁻ <i>dcm ompT hsdS gal λ</i> (DE3); T7 polymerase gene under the control of the <i>lacUV5</i> promoter	Laboratory collection
Plasmids		
pBluescript	Ap ^r	Stratagene
pT7-5	T7Φ10; Ap ^r	33
pT7-RhiE	pT7-5 bearing <i>rhiE</i>	This work
Phages		
ΦEC2	<i>E. chrysanthemi</i> generalized transducing phage	23
MudI1681	Mu <i>cts62</i> derivative (<i>lacZYA</i> Km ^r)	6

control of the T7 φ10 promoter, was not modified by this mutation (Fig. 2). It is possible that one of two in-frame ATG codons (positions 426 and 432), preceded by a putative Shine-Dalgarno sequence (AAAGA), could be the initiation codon.

Mutation of either one of them (to ATC and ATT, respectively) did not abolish RhiE synthesis, indicating that both act as initiation codons (Fig. 2). We showed that both of them had to be mutated to prevent RhiE synthesis (Fig. 2). Since RhiE

TABLE 2. Presence of RhiE in several *Erwinia* strains

Strain	Host	Geographic origin	RFLP group ^a	Growth on rhamnose	Presence of RhiE ^b
<i>E. chrysanthemi</i>					
3937	Chrysanthemum	France	NA ^c	–	+
ET3	Tomato	Martinique	33	–	+
4073	Carnation	Great Britain	NA	–	–
1240	Carnation	Denmark	33	–	–
SF109-1	Sunflower	France	43	–	+
4072	Corn	India	NA	–	–
1271	Corn	Egypt	42	–	–
4040	Potato	Peru	NA	–	–
722	Tomato	France	NA	–	–
1270	Parthenium	Denmark	NA	–	–
4062	Agalomena	St. Lucia	46	–	+
SH230	Tomato	Cuba	44	–	+
1342	Chrysanthemum	Italy	NA	–	+
1891	Tobacco	United States	39	–	–
4060	Begonia	The Netherlands	NA	–	–
4061	Colocasia	Solomon Islands	34	–	+
4065	Banana	Colombia	NA	–	–
3367	Dahlia	France	NA	–	–
3716	Kalanchoe	France	NA	–	+
CIP366	Potato	Peru	NA	–	–
ENA49	Diffenbachia	Brazil	NA	–	+
EC16	?	?	NA	–	+
<i>E. carotovora</i> subsp. <i>odorifera</i> 56–487				+	–
<i>E. carotovora</i> subsp. <i>betavascularum</i> 56–479				+	–
<i>E. carotovora</i> subsp. <i>carotovora</i> SCRI193				–	–
<i>E. carotovora</i> subsp. <i>atroseptica</i> SCRI31				–	–
<i>E. carnegiani</i> 55–483				+	–
<i>E. cyripedi</i> 51–440				–	–
<i>E. rhapontici</i> 49–421				–	–

^a The RFLP groups were defined by Waleron et al. (37).

^b The presence of RhiE was determined by immunodetection in supernatants of culture grown in the presence of rhamnose.

^c NA, not applicable.

is a secreted protein, we looked for the presence of a signal sequence. The N-terminal part of the protein is hydrophobic and contains a putative signal peptidase cleavage site (ATG/A) at position 27 from the first methionine residue (position 426) (Fig. 2). This cleavage site was confirmed by sequencing the N terminus of the RhiE protein purified from rhamnose-induced *E. chrysanthemi* culture supernatant. Labeling of RhiE expressed in *E. coli* showed two forms of the protein (Fig. 2). Treatment of the cells with carbonyl cyanide *m*-chlorophenyl-

hydrazone, which blocks signal sequence processing, led to the synthesis of only the larger form (Fig. 2). The smaller form was extractable from the *E. coli* periplasm (data not shown). Thus, the larger form corresponds to the nonmatured form of RhiE. The inefficient cleavage of the RhiE signal sequence in *E. coli* could result in the unusual presence of three prolyl residues in this sequence. Mature RhiE is a 551-amino-acid protein with a calculated molecular mass of 61,940 Da.

The sequence of RhiE was compared with sequences in databases. The best homologies were found with several proteins of unknown function in *Arabidopsis thaliana* (35). These proteins have been classified in family 4 of the polysaccharide lyases by Coutinho and Henrissat (10). This family contains two other proteins. Only one of them, produced by *A. aculeatus*, has been biochemically characterized as a rhamnogalacturonate lyase (18). Homology between these proteins was restricted to their C-terminal parts.

RhiE is secreted by the Out pathway. The presence of an N-terminal signal sequence suggested that RhiE secretion occurs via the type II Out pathway, since secretion through type I (Prt) or III (Hrp) secretion pathways is Sec independent (29). The absence of RhiE in the supernatant of an OutC mutant confirmed this hypothesis (Fig. 3). Two of the Out proteins, OutC and OutD, were shown to contain a secretion targeting signal for proteins secreted by the Out secretion machinery (5).

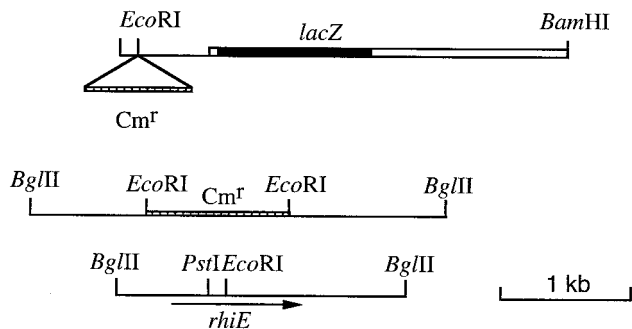


FIG. 1. Cloning of *rhiE*. The thick line represents MudII1681 DNA, and the thin line represents *E. chrysanthemi* chromosomal DNA. The hatched box represents the Cm^r cassette.

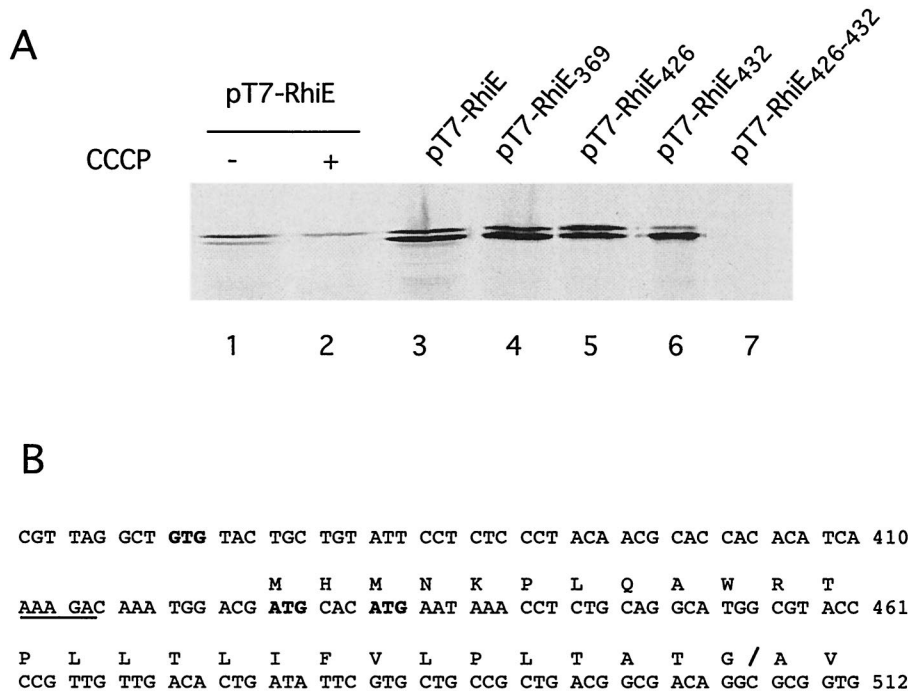


FIG. 2. Determination of the RhiE initiation codon. (A) Wild-type *rhiE* or *rhiE* mutated at putative initiation codons was cloned in the pT7-5 vector. The proteins expressed were labeled with [³⁵S]cysteine-[³⁵S]methionine and separated by SDS-PAGE, and the gels were autoradiographed. Lanes 1 and 3, BL21/T7-RhiE; lane 2, BL21/T7-RhiE treated with carbonyl cyanide *m*-chlorophenylhydrazine; lane 4, BL21/T7-RhiE₃₆₉ (M369V); lane 5, BL21/T7-RhiE₄₂₆ (M426I); lane 6, BL21/T7-RhiE₄₃₂ (M432I); lane 7, BL21/T7-RhiE₄₂₆₋₄₃₂ (M426I M432I). (B) Nucleotide sequence of the *rhiE* translation initiation region. Putative initiation codons are in boldface. The Shine-Dalgarno sequence is underlined. The protein sequence is indicated above the nucleotide sequence. The signal sequence cutting site is indicated by a slash. The numbering is that for the nucleotide sequence deposited under accession number AJ438339.

The presence of the C-terminal PDZ domain of OutC is required for the secretion of most of the Out-secreted proteins, except for Cel5, PelI, and PemA. Introduction into a $\Delta outD$ *E. chrysanthemi* mutant of an OutD protein from *Erwinia carotovora* or a hybrid containing the N-terminal domain of *E. carotovora* OutD and the C-terminal domain of *E. chrysanthemi* OutD allowed for the secretion of only PemA and PelI, indicating that the N-terminal domain of *E. chrysanthemi* OutD contains the information necessary for secretion of most of the Out-secreted proteins. Ten percent of RhiE was secreted from

the mutant A3981 (*outC* Δ PDZ), and no RhiE was secreted by the *outD* mutant strain A3657 containing plasmid pTdB-EcaOD encoding *E. carotovora* OutD (Fig. 3). This indicates that RhiE secretion is dependent on the presence of the PDZ domain of OutC and requires the *E. chrysanthemi* OutD N-terminal domain. Thus, RhiE specificity towards the Out machinery components is similar to that of most of the Out-secreted proteins.

Several studies have shown the necessity of disulfide bond formation in secreted proteins for their recognition by the secretion machinery (22, 31). However, these studies, performed with *dsb* mutants, have been weakened by the facts that the absence of disulfide bonds in secreted protein makes them unstable and that *dsb* mutations could also affect the secretion machinery. RhiE is the first characterized *E. chrysanthemi* protein which is secreted by the Out pathway but contains no disulfide bond. We studied RhiE secretion in a *dsbA dsbC* mutant, in which disulfide bonds are not formed in periplasmic proteins. In this mutant, RhiE was stable and accumulated in the cells (Fig. 3). This indicates that the Out machinery is not functional in the *dsbA dsbC* mutant.

Regulation of *rhiE* expression. To study *rhiE* expression, a transcriptional fusion was constructed. A *uidA-kan* cassette was introduced into the unique *EcoRI* site of *rhiE*. After introduction into *E. chrysanthemi*, the construction was recombined into the chromosome by successive cultures in low-phosphate medium. The absence of RhiE in the culture supernatant confirmed the replacement of the wild-type gene by the *uidA*

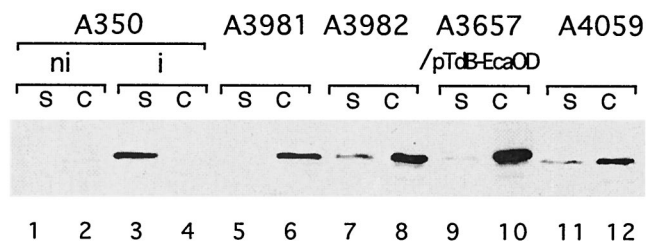


FIG. 3. Cellular localization of RhiE in various *E. chrysanthemi* strains. Bacteria were grown in LB medium in the presence of rhamnose (except for lanes 1 and 2). Proteins of the cell fraction (lanes C) and the supernatant (lanes S) were separated by SDS-PAGE and blotted onto a membrane, and the presence of RhiE was detected with anti-RhiE antibodies. Lanes 1 and 2, noninduced A350 (ni); lanes 3 and 4, rhamnose-induced A350 (i); lanes 5 and 6, A3981(*outC*); lanes 7 and 8, A3982 (*outC* Δ PDZ); lanes 9 and 10, A3657(pTdB-EcaOD); lanes 11 and 12, A4059 (*dsbA dsbC*).

TABLE 3. *rhiE* expression in various backgrounds and under various induction and growth conditions

Growth conditions ^a	Additional mutation	Carbon source	Inducer ^b	β -Glucuronidase activity ^c (mean \pm SD)	
Standard	None	Glycerol	-	7 \pm 2	
			+	1,195 \pm 190	
		Glucose	-	<1	
			+	202 \pm 41	
	<i>crp</i>	Glucose	-	<1	
			+	10 \pm 2	
	<i>kdgR</i>	Glycerol	-	8 \pm 1	
			<i>pecS</i>	Glycerol	-
<i>pecT</i>					Glycerol
Low osmolarity	None	Glycerol	+	654 \pm 108	
High osmolarity	None	Glycerol	+	825 \pm 49	
37°C	None	Glycerol	+	1,203 \pm 94	

^a Standard growth conditions were obtained by cultivation in M63 medium at 30°C with shaking at 200 rpm. Low-osmolarity medium was four fold-diluted M63 medium. High osmolarity was obtained by adding 0.3 M NaCl to M63 medium.

^b The inducer was 0.2% rhamnose.

^c β -Glucuronidase activity is expressed as nanomoles of *p*-nitrophenol formed per minute per milligram (dry weight) of bacteria.

mutant allele in strain A3946. Expression of the fusion was strongly induced in the presence of rhamnose (>200-fold) (Table 3). However, the concentration of rhamnose necessary to reach the fully induced level was very high (>2 g/liter), which suggests that rhamnose does not enter the bacteria by a high-affinity transport system but rather by passive diffusion or by a nonspecific transport system. The induction of *rhiE* by other sugars present in pectin was tested. None of the sugars (galacturonate, galactose, xylose, fucose, ribose, and arabinose) was found to be an inducer of *rhiE* expression. The effect of regulatory mutations that affect pectinase gene expression was analyzed. While a mutation in *kdgR* or *pecS* did not modify *rhiE* expression, a mutation in *pecT*, which controls expression of all of the pectate lyase genes, led to a 1.6-fold increase in *rhiE* expression (Table 3). Mutations in these three regulators did not change the rhamnose-induced induction level (data not shown). In the presence of glucose as the sole carbon source, *rhiE* expression is reduced, which suggests a possible role of CRP as an activator of *rhiE*. This was confirmed when *rhiE-uidA* was introduced into a *crp* mutant: the fusion expression level was reduced 100-fold in the presence of rhamnose. *rhiE* expression increased twofold at the end of the exponential growth phase but was not modified in an *expI* mutant that no longer synthesizes the *E. chrysanthemi* major acyl-homoserine lactone (data not shown). The effects of various environmental conditions were tested. In contrast to what is observed for the pectate lyase genes, *rhiE* expression was not reduced at high temperature (37°C). However, it was reduced at high or low osmolarity of the medium.

Occurrence of RhiE in other *Erwinia* strains. The supernatants of rhamnose-induced cultures of 21 strains of *E. chrysanthemi* and other *Erwinia* species (*E. rhapontici*, *E. cyripedi*, *E. carnegiani*, *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp.

atroseptica, *E. carotovora* subsp. *odorifera*, and *E. carotovora* subsp. *betavascularum*) were examined for the presence of RhiE. Material reacting with the anti-RhiE antibody was detected in one-third of the *E. chrysanthemi* strains and in none of the other *Erwinia* strains, indicating that a homologue of *rhiE* is not present, or is not expressed, under these conditions in these strains (Table 2). No correlation could be found between the expression of RhiE, the geographical or plant origin of the *Erwinia* strains, their RecA restriction fragment length polymorphism (RFLP) group (37), and their ability to grow with rhamnose as the sole carbon source.

Characterization of RhiE activity. The homology of RhiE with a family of proteins which includes a rhamnogalacturonate lyase led us to suppose that this protein could possess the same activity. We tried to detect lyase activity by measuring an increase in absorbance at 230 nm with several substrates (polygalacturonate, pectin, RG-I, and arabinogalactan) in the presence of an extract of *E. coli* overproducing RhiE. We could detect activity only with RG-I, confirming that RhiE is a rhamnogalacturonate lyase which cleaves the RG-I backbone between a rhamnose and a galacturonate and creates an unsaturated bond between carbons 4 and 5 of the galacturonate residue. RhiE activity was not increased by the addition of 0.2 mM Ca²⁺, Mn²⁺, Co²⁺, Cu²⁺, or Zn²⁺ and was not modified by the addition of 2 mM EDTA. The optimum pH of activity was 6.0. A possible role of RhiE in *E. chrysanthemi* pathogenicity was investigated by comparing the length of rotted tissue produced by the wild-type strain and by a *rhiE* mutant on chicory leaves. A significant decrease in the length of rotted tissue (13 \pm 5 versus 26 \pm 8 cm) showed that *rhiE* is required for the full virulence of *E. chrysanthemi* strain 3937. The purified enzyme was not able to provoke maceration of potato tubers or chicory leaves.

DISCUSSION

The plant-pathogenic bacterium *E. chrysanthemi* synthesizes and secretes a full range of enzymes that are able to degrade the smooth region of pectin, whose synthesis is induced by PGA or galacturonate. To identify enzymes able to depolymerize the backbone of RG-I, we analyzed the supernatants of rhamnose-induced cultures. The rhamnogalacturonate backbone of pectin can be cleaved by rhamnogalacturonate rhamnohydrolases and rhamnogalacturonate lyases, which cleave the α -1,4 linkage, or by rhamnogalacturonate galacturonohydrolases, which cleave the α -1,2 bond. Several of these enzymes can be produced in the same organism, as described for *A. aculeatus* (16, 17, 18). Only one major protein, RhiE, which belongs to the rhamnogalacturonate lyase family, was detected in *E. chrysanthemi* supernatants. Thus, in contrast to the case for the multiprotein family of pectate lyases, only one rhamnogalacturonase seems to be produced by this bacterium to degrade the RG-I backbone. Other possible explanations for the absence of detection of other rhamnose-induced secreted proteins is that they are synthesized in small amounts or that the induction conditions used did not allow for their synthesis. The gene *rhiE* does not seem to be present in all of the strains tested. The absence of rhamnose metabolism by *E. chrysanthemi* could render useless, and energetically costly, the complete degradation of the rhamnogalacturonate part of pectin

into monosaccharides, which cannot subsequently be used as a carbon source. The absence of *rhiE* in strain A350 reduces the virulence of the bacterium on chicory leaves. Even if RhiE is not useful in providing carbon sources for the bacterium, its enzymatic activity could be required to liberate pectin smooth regions and increase their accessibility to pectate lyase action. *E. chrysanthemi* pathogenicity is not due to a single determinant but results from the addition of several factors that lead to bacterial cell wall degradation. The presence of RhiE in some strains could complete their arsenal of degrading enzymes and increase their virulence.

RhiE regulation was compared with that of other pectinases. The main inducing condition seems to be the presence of rhamnose, which does not induce the synthesis of the pectate lyases. Among the specific regulators of the pectate lyase genes tested, only *pecT* weakly controls *rhiE* expression. Besides the genes of all of the pectate lyases, *pecT* also regulates the expression of other virulence factors, such as the synthesis of exopolysaccharides (7). *rhiE* is also activated by CRP, the main activator of virulence factor genes in *E. chrysanthemi* (24). Thus, in addition to a specific regulation by rhamnose, *rhiE* is included in the regulatory network that controls *E. chrysanthemi* virulence factors.

In contrast to other Out-secreted proteins studied up to now, RhiE does not contain any cysteine residue. This led to a reexamination of the role of disulfide bond formation in Out secretion. RhiE accumulated in the periplasm of a *dsbA dsbC* mutant, unable to form disulfide bonds in the periplasmic proteins. This nonfunctionality of the Out machinery could result from the absence of disulfide bond formation in two proteins, OutS and OutK (reference 22 and unpublished results). However, results obtained with the cellulase Cel5 mutated at cysteine residues showed that disulfide bond formation is required for the secretion of Cel5 by *E. chrysanthemi* (4). Thus, the formation of disulfide bonds is required to stabilize not only the secreted proteins but also the secretion machinery.

RhiE does not require Ca^{2+} or other divalent cations for its activity, and its optimum pH is around 6.0. These properties are similar to those of *A. aculeatus* rhamnogalacturonate lyase and of pectin lyases (18, 36) and differ from those of *P. cellulosa* rhamnogalacturonate lyase and of pectate lyases, which have an absolute requirement for divalent cations (most frequently Ca^{2+}) and are active at basic pH (14, 34). Rhamnogalacturonate lyases are found in two families of polysaccharide lyases in the classification of Coutinho and Henrissat (10). *P. cellulosa* rhamnogalacturonate lyase belongs to family PL11 (14), which contains only bacterial proteins. RhiE is the first bacterial rhamnogalacturonate lyase characterized in the PL4 family, which contains the *A. aculeatus* rhamnogalacturonate lyase (18). The *A. thaliana* MYST proteins, which also belong to family PL4, were thought to be dicotyledon-specific proteins and have no characterized activity (35). These proteins are probably also rhamnogalacturonate lyases. Thus, PL4 could be a widespread family of proteins with rhamnogalacturonate lyase activity found in plants, fungi, and bacteria. Rhamnogalacturonate lyases of PL4 and PL11 differ in their catalytic properties (pH and ion requirements), and further analysis is required to understand the basis of these differences.

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