The *exuT* Gene of *Erwinia chrysanthemi* EC16: Nucleotide Sequence, Expression, Localization, and Relevance of the Gene Product

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Accepted 12 December 1997.

Galacturonic acid (GalUA) is a major component of pectin and polygalacturonic acid in the plant cell wall. In the phytopathogen *Erwinia chrysanthemi*, the uptake of molecules derived from degradation of these polymers is an important early step in the events preceding induction of pectinases, ultimately leading to plant tissue maceration. Uptake systems for GalUA and dimers of GalUA have been described and shown to be inducible in *E. chrysanthemi*. The GalUA uptake gene (*exuT*) was cloned and sequenced. Nucleotide sequence analysis identified an open reading frame encoding a 345-amino-acid polypeptide with a calculated mass of 37,825 Da. This polypeptide is predicted to be an integral membrane protein based on its high nonpolar amino acid content and hydropathic profile. Localization studies with the labeled polypeptide in the T7-RNA polymerase system also suggest that ExuT is a membrane protein. This evidence is further supported by the observation of hybrid ExuT-PhoA proteins in the bacterial cytoplasmic membrane following immunoblot analysis. Northern (RNA) analysis indicated that the gene is inducible in the presence of the monomer, GalUA. A targeted mutation in the *exuT* gene affected the utilization of GalUA as a sole carbon source for growth. Maceration of potato tuber tissue by this mutant was delayed and reduced, when compared with the parental strain EC16.

Additional keywords: plant disease.

The production of plant cell wall-degrading pectate lyases and hydrolases by many phytopathogenic members of the genus *Erwinia* is essential for the maceration of plant tissue (Barras et al. 1994). Maceration involves the depolymerization of pectin and pectate, yielding monomers, saturated and unsaturated dimers, trimers, and oligomers of galacturonic acid (GalUA) (Starr and Chatterjee 1972; Kotoujansky 1987).

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Nucleotide and/or amino acid sequence data are to be found at the GenBank data base as accession number U35884.

Many products from degradation of these polymers are known to be catabolized intracellularly by *E. chrysanthemi* to generate energy and molecules for the induction of pectate-degrading enzymes (Collmer and Keen 1986; Barras et al. 1994; Hugouvieux-Cotte-Pattat et al. 1996). Prior to intracellular catabolism, monomers, dimers, and presumably oligomers of GalUA with a low degree of polymerization must be taken up into the bacterial cell. In *E. chrysanthemi*, the uptake system for GalUA has been studied with [¹⁴C]glucuronate (GalUA is a competitive inhibitor of GalUA) and [¹⁴C]GalUA (Hugouvieux-Cotte-Pattat et al. 1983; San Francisco and Keenan 1993). This uptake system was first discovered as part of hexuronate metabolism in *Escherichia coli* (Portalier et al. 1980). Previous genetic studies with *E. chrysanthemi* and *E. coli* have identified *exuT* as being necessary for the uptake of GalUA (Portalier et al. 1980; van Gijsegem and Toussaint 1983; Hugouvieux-Cotte-Pattat et al. 1996). The gene has not been cloned functionally in vitro, nor has the gene product been definitively localized in the bacterial cell.

The importance of intracellular inducers for the production of pectinases has been well documented in *E. chrysanthemi* (Nasser et al. 1994; Hugouvieux-Cotte-Pattat et al. 1996). These inducers are formed due to intracellular catabolism of GalUA, and dimers and oligomers of GalUA. The uptake of these molecules into the bacterial cell is a necessary prerequisite for intracellular catabolism. To extend our studies on the GalUA uptake system of *E. chrysanthemi*, the *exuT* gene was cloned and its nucleotide sequence determined. Plasmids bearing *exuT* were capable of rescuing mutants unable to take up GalUA. The polypeptide encoded by this gene was expressed in *E. coli* in the T7-RNA polymerase expression system and localized to the membrane fraction. Hybrid proteins generated by *exuT*-TnphoA gene fusions were localized to the cytoplasmic membrane fraction. Analysis of the deduced amino acid sequence also supports the observation that ExuT is a membrane protein. A mutation in the *E. chrysanthemi* EC16 *exuT* gene resulted in a reduced rate of GalUA utilization and in reduced plant tissue maceration.

RESULTS AND DISCUSSION

Nucleotide sequence analysis of the *exuT* gene.

Previous studies have identified a 3.36-kb *E. chrysanthemi* EC16 genomic fragment capable of rescuing the GalUA up-

take and growth defects in the *exuT* mutant *E. chrysanthemi* ERH215 (Freeman and San Francisco 1994). Nucleotide sequence analysis of this fragment revealed the presence of two potential open reading frames. Based on sequence homology studies with the *E. coli* genome and potential membrane-spanning domains predicted from hydropathic analysis, one open reading frame was presumed to encode ExuT. The deduced amino acid sequence of the second open reading frame predicted the occurrence of a soluble protein with a DNA binding motif. This polypeptide is presumed to encode the transcriptional repressor, ExuR (Freeman 1996). DNA encoding the putative *exuT* gene was subcloned and was capable of rescuing the [¹⁴C]-GalUA uptake defect in *E. chrysanthemi* ERH215.

The nucleotide sequence of *exuT*, including the regulatory and structural features, is shown in Figure 1. The open reading frame for the gene starts at base 459 and extends for 1,034 nucleotides. The presumed ribosome binding site GGAGA is located 10 nucleotides upstream from the ATG codon (Shine and Dalgarno 1974). A potential cAMP-CRP (cyclic AMP-cAMP receptor protein) binding site, 5'-TTTGTGATATCACTCAACTTT-3', is located between nucleotides 155 and 176 (De Crombrughe et al. 1984). Previous studies on this organism have shown that glucose exerts repression on GalUA uptake activity (Hugouvieux-Cotte-Pattat et al. 1983; San Francisco and Keenan 1993). The presence of this cAMP-CRP binding site provides a possible explanation for this glucose-repressible phenotype.

Regulation of the GalUA uptake and utilization genes (*uxaC,B,A*) is moderated by ExuR. This regulatory protein is thought to function by exerting negative control over these genes (van Gijsegem et al. 1985). No other proteins have been shown to regulate *exuT* and *uxaC,B,A* genes. KdgR is a well-characterized, negative regulatory protein that plays a major role in expression of genes encoding pectate-degrading enzymes as well as the genes involved in secretion of pectinases (Reverchon et al. 1991). A potential KdgR box, 5'-(AAT-A-AAA-T)A(A-T-G-GTT-T-T)-3', is found between bases 142 and 158 of *exuT*, partially overlapping the cAMP-CRP binding site. This KdgR binding sequence is almost identical to the proposed consensus KdgR binding sequence, (AAT-G/A-AAA-C/T)N(N-T/C-G/T-TTT-C/T-A) (Nasser et al. 1994). The six highly conserved adenines on both strands and the four symmetrical guanine residues suggest that this regulatory sequence may interact with the KdgR repressor in this organism. The observation of a potential KdgR binding sequence suggests the functioning of an additional repressor in the expression of *exuT*, but this remains to be confirmed. Indeed, this sequence may not have any relevance in vivo, as has been shown for several other *E. chrysanthemi* genes, *kdgC*, *kduD*, *pem*, *kdgA*, and *outC* (Nasser et al. 1994). The nucleotide sequence of the intervening region between *uxaC* and *exuT* has been determined previously only in *E. coli* (Blanco and Mata-Gilsinger 1986). Analysis of this region in *E. coli* revealed the presence of two cAMP-CRP binding sites but no evidence was found for a KdgR binding site. In *E. chrysanthemi*, both the cAMP-CRP and KdgR binding sites are present just upstream of the potential -10 and -35 transcriptional start sites. Commencing at nucleotide 356, 103 nucleotides upstream of the start codon of the *exuT* gene lies an inverted repeat, 5'-CGGGGCAN6TGC CCCG-3'. This inverted repeat has the potential of forming a stem and loop structure with a calcu-

lated ΔG of -19.4 kcal/mole (Tinoco et al. 1973). The observation of similar inverted repeats capable of forming stable stem and loop structures upstream of gene sequences has been made with the arsenic efflux pump (ArsB), which is an integral membrane protein (Chen et al. 1986). Such structures probably serve to limit the number of transcripts encoding integral membrane proteins to maintain an optimum lipid-protein ratio.

The *exuT* gene encodes a translational product corresponding to a protein of 345 amino acids with a calculated molecular mass of 37,825 Da. The high proportion of nonpolar amino acids (57%) and the hydropathic profile indicate that ExuT has significant hydrophobic character consistent with known integral membrane proteins (Engelman et al. 1986). Further nucleotide and protein sequence analyses were carried out with the BLAST program for nucleotides and proteins (Altschul et al. 1990). Extensive nucleotide sequence homology exists between the regions encoding the *exuT* gene of *E. chrysanthemi* and a region of the *E. coli* genome encoding the GalUA uptake gene (data not shown). This region of the *E. coli* genome has been identified genetically (Portalier et al. 1980). The *exuT* gene of *E. chrysanthemi* shows 77% homology over 996 bp to its *E. coli* counterpart. The deduced amino acid sequence is 83% identical to the presumptive translated product of the *E. coli exuT* gene.

Expression of *exuT* and localization of the gene product.

Previous studies have shown that GalUA uptake is inducible in the presence of PGA, (GalUA)₂, and GalUA (San Francisco and Keenan 1993). RNA prepared from cells grown in the presence of GalUA possessed a much greater abundance of transcripts that hybridized to the *exuT* gene probe, compared with RNA from cells grown on glycerol (Fig. 2). This suggests that *exuT* expression may be regulated at the level of transcription. The apparent transcript size is 1.2 kb, sufficient to encode a 37-kDa polypeptide. It has been presumed in the past, but never demonstrated, that GalUA uptake in *E. chrysanthemi* or any organism is mediated by a single polypeptide. Expression studies with the *exuT*-encoding fragment cloned into vector pT7-5 of the T7-RNA polymerase expression system were aimed at identifying the number and location of polypeptides encoded by the DNA. To unequivocally identify the gene product(s) encoded by this DNA, a kanamycin resistance gene was inserted into the unique *Pst*I site of the gene. This in vitro-disrupted construct was also cloned into expression vector pT7-5. Visualization of expressed proteins on polyacrylamide gels clearly demonstrates that *exuT* encodes a single polypeptide with an apparent molecular mass of 37 kDa. This matches the predicted molecular mass of the protein from the deduced amino acid sequence. Figure 3 shows an autoradiograph of [³⁵S]methionine-labeled proteins separated on a 12% polyacrylamide gel. A polypeptide with an apparent molecular mass of 37 kDa was always observed in the membrane fraction and whole cells of bacteria carrying the intact *exuT* gene. The protein was not observed in the cytoplasmic fraction. The relatively low amount of ExuT observed in membranes may be due to protein degradation or incomplete precipitation. Note that the preparation of membranes in the fractionation procedure does not include ultracentrifugation at 100,000 × g, which is normally required to fully precipitate the membrane fraction. In bacteria carrying the insertionally inactivated *exuT* fragment, no radioactively labeled polypeptide was observed. Thus, *exuT* encodes a 37-kDa membrane protein. The *npt* gene product was not observed to be radiolabeled in

these experiments because the orientation of this gene in the construct was opposite to the direction of the T7 promoter.

To further clarify the location of ExuT in the bacterial cell, *exuT-phoA* fusion-bearing strains were constructed. Inner membranes and outer membranes were separated on sucrose density gradients. Purity of the inner membrane fraction was confirmed by the NADH dehydrogenase assay. In all preparations, greater than 85% of the total NADH dehydrogenase ac-

tivity was found associated with the inner membrane. Equivalent amounts of total membrane and outer membrane proteins were separated on polyacrylamide gels. Immunoblot analysis with anti-BAP revealed the major portion of the hybrid ExuT-PhoA polypeptides to be present in the inner membrane fraction, when compared with the outer membrane fraction (Fig. 4). The sizes of the hybrid proteins are predicted based on the insertion loci of the transposon into *exuT*. No

TCACACCAATTATCGACAATAACCTCACATCATTTAGTGAGGTCATGATCGCAAAATGACAGAAATCCGTTTCAATTACA	80
<i>KpnI</i>	
CCATTTTACGCTTCCGACTCATCATATAGCCCTGTTACCGACCGAATTATTTTATTCCTCCCAATAAAATAATGGTtttgt	160
gaTATCACTcaactttTAAATTGGTATGACAAATTTATTTAGCGCTGCTGATGAGGGGATTCAATCTCAATATCGTCAA	240
CAAGATTGGTATTACCAACCGGAAAACAATAACGCGCGGCGGATCGTCTGCGAGCCCGCCAGTTGTGCCAGCGGCAGG	320
GCAACCGCCAGGCGGCAACTCCCGCAGGTTTGTACCGGGGCACAGAGGTGCCCCGTCACCTTTCGGCGTGGTCTACACCTA	400
CTACCGCAGACGGGTTACCGGCACTGCCGTGGTAATTAACTCGGAGAGATATTTAACATGTTCAAGATCAAAGGCTTGC	480
	M F K I K G L R
<i>KpnI</i>	#11
GCTGGTACATGATCGGACTGGTGACCATCGGTACCGTTCTGGGCTACCTGACGCGTAACGCAATTGCCGAGCGGCGCCC	560
W Y M I G L V T I G T V L G Y L T R N A I A A A A P	
<i>PstI</i>	
ACACTGCAGGAACAGCTCCATATCAGTACTCAGCAATACTCCTATATCATTTGCGGCCTATTTCAGCCTGCTACACCATCAT	640
T L Q E Q L H I S T Q Q Y S Y I I A A Y S A C Y T I M	
<i>KpnI</i>	
GCAGCCTGTTGCCGTTATGTGCTGGATGTACTGGGTACCAAAGTGGGTTACGCCATGTTCCGCATCCTGTGGGCACTGT	720
Q P V A G Y V L D V L G T K V G Y A M F A I L W A L F	
#3	
TCTGTGCCGGCACAGCGCTGGCTAACAGCTGGGGCGGCCCTGGCGGTAGCGCGCGCGCGGTAGGGATGGCGGAAGCGGCC	800
C A G T A L A N S W G G L A V A R G A V G M A E A A	
ATGATCCCCCGCGGTCTGAAAGCCAGCAGCGAGTGGTTCCCGGCCAAGGAACGTTCTGTTGCGGTGGGTTATTTCAACGT	880
M I P A G L K A S S E W F P A K E R S V A V G Y F N V	
TGGCTCATCGATTGGCGGCATGTTGGCTCCGCCCTGGTCTGTCGGCGATTTATGGCGCACAGCTGGCAAATGGCGTTCC	960
G S S I G G M L A P P L V V W A I M A H S W Q M A F L	
TGATCACCGGCGCACTGAGCCTGGTTTGGGCACTGTGCTGGCTCTACTTCTACAAGCATCCGAAAGATCAGAAAAAATC	1040
I T G A L S L V W A L C W L Y F Y K H P K D Q K K L	
#33	
AGCACCGAAGAACCGCAATATATTTCTGAGCGGTACGGAAGCACAGCATCAGGCCGGCAACGCCAAGAGAATGTCCGCCTG	1120
S T E E R E Y I L S G Q E A Q H Q A G N A K R M S A W	
GCAGATTCTGCGTAACCGTCAGTTCTGGGGCATCGCGTTGCCGCGCTTTCTGGCAGAACCGGCTGGGGTACGTTCAACG	1200
Q I L R N R Q F W G I A L P R F L A E P A W G T F N A	
CCTGGATCCCGCTGTTTCATGTTCAAGGCTATGGCTTTAACTGAAAGAAATCGCCATGTTTCGCCTGGATGCCGATGCTG	1280
W I P L F M F K A Y G F N L K E I A M F A W M P M L	
TTTCCGACCTCGGCTGTATCCTCGGCGCTACATGCCGATGCTGTTCCAGAAGTACTTCAAAGTGAACCTGATAGTCTC	1360
F A D L G C I L G G Y M P M L F Q K Y F K V N L I V S	
CCGTAAACTGGTTCGTGACGCTGGGTGCGTTGTTGATGATCGGCCCGGCACCATCGGCCTGTTACCACTGCGTATGTGG	1440
R K L V V T L G A L L M I G P G T I G L F T S P Y V A	
CTATCGCCTGCTGTGCGTTCGGCGCTTTGCCCACCACTCCTTGTCCGGTGGCTGATTAACATGTTCTCCGATGTATTCTG	1520
I A C C A S A A L P T S P C P V R *	
GCCGTAATGAAGTCGCCACCGCAATGGTTTGACCGGGATGGCCGCTGGACCGCCAGTACGCTGTTTGCTCTGGTCTGTC	1600
GGCGCTCTGGCCGACACCATCGGCTTCAGCCGTCTGTTGCGCTGTACTGGCTATCTTTGACTTGCTGGGCGCGGTCGTTA	1680
TCTGGACGGTACTGAAAAACAACCCGGTTTCCGAAGAGGGCGACAAGGCCGAGACCTGAAAGCCGCACAGCAGCACTGA	1760
<i>KpnI</i>	
CAGACAAATTTCTGCTTCACGCCCTTCATCCGGTATCATAAGGTACCGGATGAATCATTTCTTCGGCCAGGCGACAACA	1840
GCTAACTGTGACATGGGTGTTGGTTCGCCACTTGTGTAGGTGGTATAACAAATTTATTTCTGATTATCATTTCTATATACCC	1920
<i>Apal</i>	
AAGCAGTTCGAGTTGCAGGACAACATGCTCGCGTTTTGAATACAACCCATAGCGCGAGGGCCC	1983

Fig. 1. Nucleotide sequence of the *exuT* gene of *Erwinia chrysanthemi* EC16. Deduced amino acid sequence is indicated below nucleotide sequence. Proposed ribosome binding site is underscored as (*****). Potential -10 and -35 sequences are underlined and italicized. Inverted repeat sequence 5' to *exuT* is marked with arrows above sequence. Translational stop codon is indicated by (*). Potential cAMP-CRP binding site is indicated in lowercase and the adjacent, potential KdgR binding site underlined. Relevant restriction enzyme sites and transposon insertion points are noted above the sequence.

proteins from the non-fusion-bearing strains showed any reactivity with the anti-BAP antibody. These data support the contention that ExuT is an inner membrane protein.

The *exuT* mutation causes a reduction in growth on GalUA and maceration of potato tuber tissue.

To investigate the relevance of ExuT to GalUA utilization, growth studies were conducted in M9-minimal salts with GalUA as the sole carbon source. In comparison with the parental strain, the *exuT* mutant had a markedly reduced ability to grow on GalUA. Growth of the mutant after 12 h was approximately 20% that of the parent strain. Doubling time of the mutant was approximately three times longer than that of the parent strain. The ability of the mutant to grow on the monomer, although slowly, suggests the possible functioning of an alternate uptake system for the molecule. Potato maceration studies revealed a small but distinct difference in the extent of rotted tissue between the *exuT* mutant and the parent strain. Photographs taken at three time points over a 48-h period revealed that the mutant strain macerated the tissue at a slower rate and to a lesser magnitude than the parent (Fig. 5). The extent of macerated tissue was analyzed graphically at each time point and subjected to a two-tailed, repeated measures analysis of variance. Differ-

ences between the parent strain and the *exuT* mutant in the area of tissue macerated were found to be statistically significant ($P < 0.005$) at each time interval. Previous studies on plant-inducible genes in *E. chrysanthemi* 3937 identified GalUA utilization mutants as being deficient in plant pathogenicity (Beaulieu and van Gijsegem 1990). The observations in this study support those findings. Thus, it appears that *exuT* could play a role in the development of plant disease caused by *E. chrysanthemi*.

GalUA is an important component of pectin and polygalacturonic acid, which have a cementing role in the plant cell wall. The uptake of molecules derived from degradation of these polymers is an important early step in the events preceding induction of pectinases. Uptake systems for GalUA and (GalUA)₂ have been described in *E. chrysanthemi* (Collmer and Batement 1981; San Francisco et al. 1996; Hugouvieux-Cotte-Pattat et al. 1996). ExuT is probably the major transport protein that facilitates uptake of GalUA into *E. chrysanthemi*. This transport protein possibly also plays a role in maceration of plant tissue. Indeed, preliminary studies with *exuT-luxAB* fusions have suggested that the gene is expressed in the early stages on the

Fig. 2. Autoradiograph of RNA blot analysis of *exuT* transcripts. RNA was isolated from uninduced cells (U) and induced cells (I) and separated on a 1.2% formaldehyde gel. Arrow represents an approximate transcript size of 1.2 kb. The 1.1-kb internal *KpnI* fragment of the *exuT* gene was used as a probe.

Fig. 3. Expression and localization of ExuT. [³⁵S]methionine-labeled proteins were fractionated and separated on 12% sodium dodecyl sulfate-polyacrylamide gels. Radioactively labeled proteins were visualized by fluorography. Molecular mass markers (kDa) are (from top) 101, 83, 50.6, 35.5, 29.1, 20.9. Arrow indicates the ExuT protein. Lanes 1, 3, 5: cytoplasm, membrane, and whole cells, respectively, of pT7-5T carrying *exuT*. Lanes 2, 4, 6: cytoplasm, membrane, and whole cells respectively, of pT7-5K carrying *exuT* with kanamycin cassette insertion.

bacterial-plant interaction (V. Valmeekam, T. Freeman, Y. Loh, and M. J. D. San Francisco, *in preparation*). The identification of a potential KdgR binding site suggests that expression of *exuT* may be moderated by at least two repressors, KdgR and ExuR. Furthermore, the identification of a potential cAMP-CRP binding site in the 5' upstream sequence of *exuT* supports previous physiological evidence of glucose moderation of *exuT* expression. Studies currently underway in our lab are aimed at the regulation of *exuT* expression *in vivo* and *in planta*.

MATERIALS AND METHODS

Bacterial strains, phage, plasmids, growth conditions.

Strains of *E. chrysanthemi* and *E. coli*, phage, and plasmids with their genotypes and sources used in this study are listed in Table 1. *E. chrysanthemi* strains were grown at 28 to 30°C and *E. coli* at 37°C. Luria-Bertani medium (LB) or M-9 salts medium supplemented with appropriate sources of carbon, amino acids, vitamins, and trace elements were used as described by Miller (1972). Carbon sources were added to a final concentration of 0.1%. When a mixture of pectin plus po-

lygalacturonic acid (PGA) was used, each component was present at a final concentration of 0.05%. Growth rates were quantified in Klett flasks by measuring absorbance at regular intervals with a Klett-Summerson colorimeter (590 to 660 nm). Ampicillin (50 µg/ml), kanamycin (50 µg/ml), and tetracycline (20 µg/ml) were used in the growth media as noted.

Subcloning and DNA sequencing.

DNA manipulations followed standard procedures (Sambrook et al. 1989). A 3.36-kb *EcoRV* fragment of DNA from *E. chrysanthemi* EC16 was identified as being capable of rescuing the *exuT* mutation in *E. chrysanthemi* ERH215 (Freeman and San Francisco 1994). The nucleotide sequence of both strands of this fragment was determined by the dideoxy chain termination procedure with [³⁵S]dATP (Sanger et al. 1977). Sequencing primers were obtained from the Texas Tech University, Institute for Biotechnology Core Facility, and ACGT Inc. Analysis of the DNA sequence was carried out with the Genetics Computer Group, Wisconsin, DNA sequence analysis package, version 8.0 (1994). Two open reading frames were recognized, one possibly encoding *exuT*. A 1.82-kb *EcoRI*-*ApaI* fragment was derived from the 3.36-kb fragment by digestion with *ApaI* and *HindIII*, blunt ending, and religation to generate pTTU-4. This subcloned DNA was found to be sufficient to rescue the GalUA transport defect in *E. chrysanthemi* ERH215. The *exuT-uxaC* intergenic region of *E. chrysanthemi* EC16 was investigated with the 5' *EcoRI*-*PstI* fragment from pTTU-4 as a probe against the genomic library of the bacterium. This screen revealed an additional 163 bp of DNA belonging to the 5' terminus of *exuT*. This region is similar to the *exuT-uxaC* intergenic region of *E. coli* (Blanco and Mata-Gilsinger 1986). The GenBank accession number for *exuT* is U35884.

Northern blot hybridization.

Bacteria were grown under inducing (GalUA), and non-inducing (glycerol), conditions to approximately 100 Klett units and harvested to isolate total RNA. Equal amounts (10 µg) were separated by denaturing formaldehyde gel electrophoresis (Sambrook et al. 1989). Northern transfer and hybridization were performed with the *exuT* gene labeled with [α -³²P]dCTP as a probe (Sambrook et al. 1989).

T7-RNA polymerase expression of the *exuT* gene.

The *EcoRI*-*ApaI* fragment of pTTU-1 was cloned into pT7-5 to facilitate expression of its gene product by the T7-RNA polymerase expression system (Tabor and Richardson 1985). For control experiments, the *nptI* gene (*km^r*) from plasmid pUM24 was inserted into the unique *PstI* site of *exuT* cloned into pT7-5. Expression studies were carried out in *E. coli* HB101 containing the gene for T7-RNA polymerase under the control of a temperature-sensitive repressor on plasmid pGP1-2. Cells were grown and polypeptides labeled with 50 µCi [³⁵S]methionine (1,000 Ci/mmol, New England Nuclear, Trans-Label, Boston) as described. Radioactively labeled cells were fractionated into membranes and cytoplasm (Manoil and Beckwith 1985). Each fraction was assayed for incorporation of radioactivity by scintillation counting of TCA-insoluble material and electrophoresed on reducing 12% sodium dodecyl sulfate-polyacrylamide gels. The labeled proteins were visualized on X-ray film by fluorography with ENH³ANCE (Dupont-NEN, Boston) at -80°C for 48 h.

Fig. 4. Localization of ExuT-PhoA hybrid proteins. Immunoblot analysis of independent fusions fractionated and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, using anti-BAP antiserum. Molecular mass markers (kDa) are (from top) 101, 83, 50.6, 35.5, 29.1. Lanes 1, 3, 5, 7: inner membranes of insertion #3 (AP⁻), insertion #11 (AP⁺), insertion #33 (AP⁺), pTTU-4 (negative control). Lanes 2, 4, 6, 8: outer membranes of insertion #3 (AP⁻), insertion #11 (AP⁺), insertion #33 (AP⁺), pTTU-4 (negative control). Hybrid proteins are indicated with arrows.

Generation and analysis of *TnphoA* gene fusions into *exuT*.

Gene fusions were made into pTTU-4 with transposon *TnphoA* introduced through a lambda vector as described (Manoil and Beckwith 1985; Wilmes-Riesenberg and Wanner 1992). Alkaline phosphatase positive (blue) colonies on 50 µg of 5-bromo-4-chloro-3-indolyl phosphate (XP) per ml were individually picked. Plasmids containing transposons in the cloned DNA were used in studies to localize the hybrid pro-

teins. Transposon insertion loci were identified by restriction enzyme analysis.

Inner (cytoplasmic) and outer membranes of cells carrying fusions of interest were prepared by lysis of spheroplasts in a French-pressure cell followed by ultracentrifugation and sucrose density-gradient centrifugation (Osborn et al. 1972). The NADH dehydrogenase assay was used to determine the purity of the inner membrane fraction. Membrane proteins (30 µg per sample) were separated on reducing 10% sodium dodecyl sulfate-

Fig. 5. Effects of the *exuT* mutation on tissue maceration potential of *Erwinia chrysanthemi*. Potato tubers were inoculated with 10^8 cells and incubated as described. Representative photographs for two of the 15 replicates taken at 16-, 24-, and 48-h time points are shown from left to right. Parent strain (rows A and C); *exuT* mutant (rows B and D).

Table 1. Strains, plasmids, and phage used in this study

Strain	Genotype	Source/reference
<i>Escherichia coli</i>		
DH5α	F'F80dlacZ ΔM15 Δ(lacZYA-argR) U169 deoR recA endA1 hsdR17 (rK ⁻ , mK ⁺)	Gibco-BRL, Gaithersburg, MD
HB101	supE44 hsdS20 recA13 ara-14 proA2 lacY1 GallK2 rpsL20 xyl-5 mtl-1	Sambrook et al. 1989
CC118	araD139 Δ(ara, leu) 7697 ΔlacX74 phoAD20	R. Taylor, University of Tennessee
<i>Erwinia chrysanthemi</i>		
EC16	Wild type	A. K. Chatterjee, University of Missouri
ERH215	<i>E. chrysanthemi</i> B374 <i>exuT1 kdga</i> ⁺	F. van Gijsegem, INSA, Lyon
SF1601	<i>E. chrysanthemi</i> EC16 <i>exuT</i> Kan ^r	This study
Plasmids		
pRK415	pRK404 derivative Tet ^r	A. K. Chatterjee, University of Missouri
pTTU-1	3.36 kb <i>EcoRV</i> fragment from EC16 in pUC19	This lab
pTTU-4	1.82 kb <i>EcoRI</i> - <i>ApaI</i> carrying <i>exuT</i> in pUC19	This study
pTTU-5	1.82 kb <i>EcoRI</i> - <i>ApaI</i> carrying <i>exuT</i> in pUC19	This study
pTTU-6	1.82 kb <i>EcoRI</i> - <i>ApaI</i> carrying <i>exuT</i> in pRK415	This study
pTTU-7	pTTU-6 with kanamycin gene insert	This study
pGP1-2	T7-RNA polymerase gene controlled by λP _L promoter	S. Tabor, Harvard University
pT7-5	Expression vector with T7-RNA polymerase promoter	S. Tabor, Harvard University
pT7-5T	1.82-kb <i>EcoRI</i> - <i>ApaI</i> carrying <i>exuT</i> in pT7-5	This study
pT7-5K	pT7-5T with Kan ^r gene inserted into unique <i>PstI</i> site	This study
pUM24	pUC4K with <i>nptI-sacB-sacR</i> cartridge	A. Collmer, Cornell University
Phage	λ <i>TnphoA</i> B221 c1857 Pam3 with <i>TnphoA</i>	R. Taylor, University of Tennessee

polyacrylamide gels, transferred to nitrocellulose membranes by electroblotting, and incubated with rabbit antibody directed against bacterial alkaline phosphatase (BAP) (Towbin et al. 1979). Bound rabbit antibody was visualized with AP-linked goat anti-rabbit antibody and reagents supplied in a commercially available detection kit (5'—>3', Inc., Boulder, CO).

Mutagenesis of the *exuT* gene.

The *exuT* gene was isolated by digesting pTTU-1 with *ApaI* and *XbaI* blunt ending and religation to yield plasmid pTTU-5. This construct was then digested with *EcoRI* and *HindIII* and the resultant 1.84-kb fragment was cloned into pRK415 to yield pTTU-6. The kanamycin gene as a *PstI* cassette from pUM24 was blunt-end ligated into the blunt-ended *Clal* site of pTTU-6, resulting in pTTU-7. Cells were transformed with pTTU-7 and selected for kanamycin resistance and tetracycline sensitivity following growth in a low-phosphate medium as previously described (Roeder and Collmer 1985). Insertion of the kanamycin gene into the chromosomal copy of *exuT* was confirmed by Southern blot hybridizations with the kanamycin and *exuT* genes as probes.

Potato maceration assay.

Potato tubers were surface sterilized with 0.1% bleach. Slices were then cut and cored to a uniform depth with a cork borer at 2 sites separated by at least 3 cm. The wells had a diameter of 3 mm. Approximately 10⁸ cells from an overnight culture grown in LB and washed twice in 1× M9 salts were inoculated into these wells. Inoculated slices were incubated in magenta boxes at 30°C in a humid chamber. Photographs of macerated tissue were taken at 16-, 24-, and 48-h intervals. The area covered by macerated tissue was determined graphically from 15 experimental replicates by video documentation. Analysis was performed on a Power Macintosh 6100 with the public domain NIH Image program (developed at the U.S. National Institutes of Health).

ACKNOWLEDGMENTS

This work was supported by NRI grant #94-3703-0547 from the U.S. Department of Agriculture to M. J. D. S. F. Support for this research was provided in part by grants from the National Science Foundation Research Experiences for Undergraduates Program (DBI-9322342) and from the Howard Hughes Medical Institute through the Undergraduate Biological Sciences Education Program. The assistance of Richard Strauss in the statistical analysis is greatly appreciated.

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