

## *Erwinia chrysanthemi tolC* Is Involved in Resistance to Antimicrobial Plant Chemicals and Is Essential for Phytopathogenesis†

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**TolC is the outer-membrane component of several multidrug resistance (MDR) efflux pumps and plays an important role in the survival and virulence of many gram-negative bacterial animal pathogens. We have identified and characterized the outer-membrane protein-encoding gene *tolC* in the bacterial plant pathogen *Erwinia chrysanthemi* EC16. The gene was found to encode a 51-kDa protein with 70% identity to its *Escherichia coli* homologue. The *E. chrysanthemi* gene was able to functionally complement the *E. coli tolC* gene with respect to its role in MDR efflux pumps. A *tolC* mutant of *E. chrysanthemi* was found to be extremely sensitive to antimicrobial agents, including several plant-derived chemicals. This mutant was unable to grow in planta and its ability to cause plant tissue maceration was severely compromised. The *tolC* mutant was shown to be defective in the efflux of berberine, a model antimicrobial plant chemical. These results suggest that by conferring resistance to the antimicrobial compounds produced by plants, the *E. chrysanthemi tolC* plays an important role in the survival and colonization of the pathogen in plant tissue.**

The bacterium *Erwinia chrysanthemi* is a gram-negative broad-host-range phytopathogen causing soft-rot disease. Production of plant cell wall-degrading enzymes and avirulence proteins by the phytopathogen contributes to the destruction of plant structural barriers and evasion of certain host defense responses, respectively (4, 9, 10, 21, 37). Nutrient acquisition in *E. chrysanthemi* from degraded plant cell-wall pectin is facilitated by transport systems for pectin-derived oligomers and monomers (18, 20, 22, 46). Secretion of pathogenesis-related proteins across the bacterial cytoplasmic and outer membranes requires several export systems that have been extensively studied. Pectin-degrading enzymes are secreted by a type II-dependent mechanism (encoded by the *out* genes), and secretion of avirulence proteins into the host is mediated by a type III-dependent process (encoded by the *hrp* genes) (9, 33, 35). Finally, secretion of metalloproteases by a sec-independent type I mechanism (*prt* genes) in *E. chrysanthemi* has also been demonstrated (11, 25, 26).

TolC is an important though low-abundance protein in the outer membrane of gram-negative bacteria (17). The crystal structure of this protein has recently been determined (24). The protein exists functionally as a trimer forming a  $\beta$ -barrel with a large internal diameter, facilitating movement of both large and small molecules through the outer membrane (2). TolC functions as a component of multidrug resistance (MDR) efflux systems in the removal of a broad range of toxic chemicals from the cell (16, 41). In *Escherichia coli*, type I-dependent secretion of certain virulence-associated proteins also requires TolC (48). The role of TolC in virulence and survival

strategies of the pathogenic enteric bacteria *E. coli* (49), *Vibrio cholerae* (5), *Salmonella enterica* serovar Enteritidis (40), and *Serratia marcescens* (6) has been established. While the existence and role of TolC in many bacterial pathogens of mammals have been established, the occurrence and role of a TolC homologue in a plant pathogenic bacterium have not been conclusively demonstrated. Here we present the characterization of the *E. chrysanthemi tolC* and demonstrate its importance in resistance to antimicrobial plant chemicals as well as phytopathogenesis.

### MATERIALS AND METHODS

**Bacterial strains and plasmids, media, and growth conditions.** The bacterial strains, plasmids, and phages used in this study are listed in Table 1. *E. chrysanthemi* and *E. coli* cells were grown (while being shaken at 200 rpm for all liquid cultures) in Luria-Bertani (LB) medium at 30 and 37°C, respectively. Antibiotics were added where required at the following concentrations unless specified otherwise: kanamycin (Kan), 50  $\mu$ g/ml; ampicillin (Amp), 50  $\mu$ g/ml; tetracycline (Tet), 10  $\mu$ g/ml; streptomycin (Str), 25  $\mu$ g/ml; and novobiocin (NOV), 1 to 20  $\mu$ g/ml. Deoxycholate (DOC) was used in LB growth medium at a final concentration of 0.1 to 10%. Plant-derived chemicals such as rhein (9,10-dihydro-4,5-dihydroxy-9,10-dioxo-2-anthracenecarboxylic acid), genistein (4',5,7-trihydroxyisoflavone), plumbagin (5-hydroxy-2-methyl-1,4-naphthalenedione), *p*-coumaric acid and *t*-cinnamic acid (phenolic acids), pyriothione (2-mercaptopyridine-1-oxide), esculetin (6,7-dihydroxycoumarin), and berberine (5,6-dihydro-9,10-dimethoxybenzo-1,3-benzodioxoloquinolizinium) were purchased from the Sigma-Aldrich Chemical Co. Using appropriate solvents, stock solutions of all the plant-derived chemicals were made at 10 mg/ml. Esculetin was dissolved in 100% methanol, berberine and pyriothione were dissolved in water, and rhein and genistein were dissolved in 50 mM NaOH, while the rest of the plant-derived chemicals were dissolved using 95% ethanol. Appropriate solvent controls were incorporated into the experiments to account for the solvent effect.

**DNA methods.** Plasmid or genomic DNA preparation and DNA purification from agarose gels were carried out using respective Qiagen kits (QIAGEN Inc). Restriction digestion, ligation, DNA electrophoresis, and *E. coli* transformations were performed following manufacturer recommendations or as described by Sambrook et al. (34). For transformation of *E. chrysanthemi*, a single colony of EC16 culture was inoculated in 2 ml of LB broth and grown while being shaken overnight at 30°C. The culture was diluted 1:50 in 50 ml of LB broth and grown

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† Dedicated to the memory of Noel Keen.

TABLE 1. Bacterial strains, phages, and plasmids

Strain or plasmid	Genotype	Source or reference
<b>Strains</b>		
<i>Escherichia coli</i>		
DH5 $\alpha$	F $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZY-argR</i> )U169 <i>deoR recA endA1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>-</sup> )	Gibco-BRL 34
HB101	<i>supE44 hsdS20 recA13 ara-14 proA2 lacY1 GalK2 rpsL20 xyl-5 mtl-1</i>	B. Bachman
LM512	Parent of LM1151	J.A. Fralick
LM1151	LM 512 <i>tolC recA</i> Str <sup>r</sup> Tet <sup>r</sup>	This study
LM1051	LM1151 carrying pLAFR1051	
<i>Erwinia chrysanthemi</i>		
EC16	Wild type	A. K. Chatterjee
SF1603	EC16 carrying a <i>nptI</i> insertion in <i>tolC</i> gene, Kan <sup>r</sup>	This study
SF1603a	SF1603 carrying pBR2316, Amp <sup>r</sup> , Kan <sup>r</sup>	This study
SF1603b	SF1603 carrying pBR2353, Amp <sup>r</sup> , Kan <sup>r</sup>	This study
<b>Plasmids</b>		
pLAFR3	Tet <sup>r</sup> <i>cos Tra</i> <sup>-</sup> <i>Mob</i> <sup>+</sup> <i>IncP</i>	11
pLAFR1051	pLAFR3 with EC16 <i>tolC</i> genomic DNA fragment	This study
pBR2316	pBR322 derivative carrying EC16 <i>tolC</i>	This study
pBR2353	pBR322 derivative carrying <i>E. coli tolC</i>	This study

while being shaken at 40°C for 6 h. The culture was centrifuged and washed three times with 20 ml of ice-cold 10% glycerol. The cell pellet was resuspended in 0.2 ml of 10% glycerol and maintained on ice. The cell suspension (0.02 ml) was mixed with 0.005 ml of plasmid DNA–0.1 to 1  $\mu$ g DNA in water, chilled on ice for 5 min, and pulsed (using a Cell-Porator with a voltage booster [BRL Life Technologies Inc.]) at 2 kV and 4 k $\Omega$ . Cells were transferred immediately to 1 ml of LB medium and grown while being shaken at 30°C for 1 h. The culture was then centrifuged, and the pellet was resuspended in 0.1 ml of LB medium. Aliquots of resuspended cells were spread on LB agar plates carrying appropriate antibiotics.

**Isolation of *E. chrysanthemi tolC*.** An *E. chrysanthemi* EC16 cosmid library in pLAFR3 vector (provided by Noel T. Keen [11]) was mated with LM1151 in the presence of a helper strain (*E. coli* HB101) containing a self-transmissible plasmid, pRK2013 (14). Following triparental mating, transconjugants were screened for restoration of DOC resistance by direct selection on LB agar plates containing 5% DOC. The cosmid clone (pLAFR1051) that conferred DOC resistance was isolated, and its functional complementation ability was confirmed by repeating the triparental mating. DOC- and Tet-resistant colonies were restreaked on selective media before further analysis.

**Subcloning and DNA sequencing.** A 5.4-kb *ClaI* and *PstI* functionally complementing fragment of the cosmid pLAFR1051 was cloned into pBluescript, subcloned into *E. coli* DH5 $\alpha$ , and sequenced. The initial partial sequence obtained was used to design internal primers to completely sequence both strands of the *E. chrysanthemi tolC* gene from pLAFR1051. The sequence information was utilized to design nucleotide primers upstream of the *E. chrysanthemi tolC* transcription start site and downstream of the translational stop codon. Using these primers, a PCR product of about 2.0 kb (full-length *tolC* gene) was obtained using *E. chrysanthemi* EC16 genomic DNA. The PCR product was cloned into pCR2.1-TOPO cloning vector (Invitrogen) and sequenced. The functional complementing ability of the PCR product was confirmed by transforming this construct into LM1151 and direct selection on LB plates containing 5% DOC. Using an ABI Prism 310 automated DNA sequencer (Perkin Elmer), all DNA sequencing was carried out at the Texas Tech University Center for Biotechnology and Genomics core facility.

**Complementation analysis.** Cosmid pLAFR1051 carrying *E. chrysanthemi tolC* was transformed into LM1151 for complementation analysis (23). Transformants were screened on appropriate antibiotics and then tested for resistance to MDR efflux pump substrates (NOV, DOC, and carbonyl cyanide *m*-chlorophenylhydrazine [CCCP]) and sensitivity to colicin E1 and bacteriophage U3. Drug sensitivity was tested using a filter disk zone inhibition assay (16). Phage or colicin sensitivity was determined by cross-streaking or spotting the phage or colicin with bacterial cells on LB agar plates and incubating overnight. The occurrence of clearing at the intersection of cross-streaks or plaques on bacterial lawns was used as an indication of phage or colicin sensitivity.

**Immunoblot analysis of TolC.** *E. chrysanthemi* outer membranes were prepared as previously described (23, 18). Following protein separation on SDS–10% polyacrylamide gels and transfer to polyvinylidene difluoride (PVDF) membranes, immunological analysis was performed (using *E. coli* anti-TolC rabbit

polyclonal antiserum) on outer-membrane preparations and detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G and 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium (Sigma Co.). Coomassie blue staining was performed according to standard procedures (34).

**Marker-exchange mutagenesis.** Marker-exchange mutagenesis was carried out as described previously (32) with few modifications. *E. chrysanthemi* EC16 carrying the *tolC::nptI* (Kan<sup>r</sup>) construct in pBR322 (Amp<sup>r</sup>) vector was grown overnight at 30°C in 2 ml of LB medium containing 25  $\mu$ g of Kan/ml. The culture was centrifuged and washed in basal A-P medium (containing, per liter, 12.1 g of Tris, 0.1 g of MgSO<sub>4</sub> • 7H<sub>2</sub>O, 1 g of NH<sub>4</sub>SO<sub>4</sub>, 0.5 g of sodium citrate, and 2 ml of glycerol, with the pH adjusted to 7.4) and resuspended in 1 ml of the same medium. Cells were diluted 1:1,000 into 1 ml of the basal A-P medium containing 1 mM potassium phosphate buffer (pH 7.4) and 15  $\mu$ g of Kan/ml and grown for 12 h at 30°C. Following this, the culture was again diluted 1:1,000 into 1 ml of the basal A-P medium containing 1 mM potassium phosphate buffer (pH 7.4) and grown for 12 h at 30°C. Appropriate dilutions (10<sup>-6</sup> to 10<sup>-7</sup>) of the culture were then plated on LB agar (agar at 1.2% concentration) containing 15  $\mu$ g of Kan/ml and then screened for Kan<sup>r</sup> Amp<sup>s</sup> colonies to obtain the EC16 *tolC* mutant.

**Zone inhibition assays.** Cultures of respective strains were grown overnight. Approximately 10<sup>6</sup> cells were mixed with 3 ml of 0.8% agar, overlaid on LB agar plates containing appropriate selection antibiotics, and allowed to solidify. Sterile paper disks (Whatman filters) 6 mm in diameter were placed on the surface of the agar. A total of 0.03 ml of NOV (20 mg/ml), SDS (10% [wt/vol]), DOC (5% [wt/vol]), or CCCP (8 mM) was pipetted onto separate disks. The plates were incubated for 36 h, and the diameters (in millimeters) of the zones of inhibition were measured.

**Determination of MIC.** MIC assays were performed in a final volume of 0.2 ml in 96-well microtiter plates. Appropriate hydrophobic agents were serially diluted in LB broth containing the appropriate selection antibiotics for the different strains. Appropriate controls for the solvents used to dissolve the hydrophobic chemicals were also included. Bacteria from an overnight culture were inoculated at a final concentration of 10<sup>6</sup> cells/ml. The microtiter plates were shaken at 200 rpm at 30°C for 36 h. The MIC was determined as the concentration of the substance which prevented growth after 36 h.

**Plant pathogenicity assay.** Heads of witloof chicory (*Cichorium intybus* L. [Belgian endive]) were purchased from a local supermarket. Cultures of appropriate strains grown overnight were centrifuged and washed twice with 5 mM MES (morpholineethanesulfonic acid), pH 6.5. Cells were resuspended in the same buffer at a density of approximately 10<sup>8</sup> cells/ml. Using a sterile toothpick, chicory leaves were inoculated by introducing 0.005 ml of inoculum into a small wound created on the inner surface of each leaf. Leaves were maintained in a moist chamber at 30°C for 72 h. Lesions were identified by browning and maceration around the site of infection. The area of macerated tissue was defined as the product of the length and width of the lesion. To estimate the total number of bacteria per infected leaf, the leaves were homogenized in 5 mM MES and serial dilutions were plated on appropriate antibiotic plates.

**Determination of berberine accumulation.** Determination of berberine accumulation was performed as described previously (39) with a few modifications.

TABLE 2. Functional analysis of *E. chrysanthemi* EC16 *tolC* in *E. coli*

Strain <sup>b</sup>	Diam (mm) of zone of inhibition <sup>a</sup> in the presence of:		
	CCCP	NOV	DOC
LM512	20	15	6
LM1151	33	28	20
LM1051	22	17	6

<sup>a</sup> Disk diameter, 6 mm.

<sup>b</sup> LM512, parent strain of LM1151; LM1151, *E. coli tolC* deletion strain; LM1051, LM1151 with pLAFR1051 and *E. chrysanthemi tolC*.

Cultures of appropriate strains grown overnight were centrifuged and washed twice with 20 mM HEPES buffer (pH 7.0). Cells were resuspended to an optical density of 600 nm of 0.3 in HEPES buffer containing 0.2% glucose and incubated at 30°C for 90 min. The cells were washed and resuspended to an optical density of 600 nm of 0.15 in HEPES buffer. Assays were performed in 96-well flat-bottom black plates (Polyfiltronics) in a final volume of 0.2 ml. Berberine was added at 0.03 mg/ml, and fluorescence was measured using a Bio-Tek FL600 microplate fluorescence reader (Bio-Tek Instruments, Inc.), with a 360/40-nm excitation filter and a 530/25-nm emission filter.

## RESULTS AND DISCUSSION

***E. chrysanthemi tolC* can functionally complement an *E. coli tolC* mutant.** TolC mutants of *E. coli* are hypersensitive to detergents such as SDS and a variety of hydrophobic agents such as DOC, NOV, and CCCP (31, 44). To screen for the presence of *E. chrysanthemi* EC16 *tolC*, the EC16 genomic library (cloned into pLAFR3) was conjugated into an *E. coli tolC* deletion strain (LM1151) by triparental mating and transconjugants were selected on LB agar plates containing DOC. This procedure allowed us to identify cosmid pLAFR1051, which conferred DOC resistance to the *E. coli tolC* mutant. To determine whether this DNA fragment complemented other *tolC* phenotypes, we examined its ability to rescue LM1151 to NOV, CCCP, and DOC resistance. Table 2 shows that the *E. chrysanthemi tolC* was able to complement the *E. coli tolC* mutant to drug resistance. Chemicals such as NOV and DOC are known to be substrates of the AcrAB/TolC efflux pump, while CCCP is known to be a substrate of the EmrAB/TolC efflux system (7, 29). Thus, it is likely that homologs of these efflux pumps in *E. chrysanthemi* can utilize TolC in conferring resistance to these substrates.

**The *E. chrysanthemi tolC* gene product bears strong identity to its *E. coli* counterpart.** Southern dot blot analysis using the *E. coli tolC* gene as a probe for the pLAFR1051 cosmid DNA and genomic DNA from *E. chrysanthemi* indicated that there was nucleotide sequence homology for this gene between the two microorganisms (data not shown). Using primers generated from the cosmid-derived partial *tolC* sequence and *E. chrysanthemi* genomic DNA as the template, PCR amplification yielded a 2-kb fragment carrying the complete *tolC* gene, which was cloned and sequenced (GenBank AF421372).

Nucleotide sequence analysis of the 2,004-base PCR fragment indicates that the *tolC* homologue from *E. chrysanthemi* encodes an open reading frame of 1,410 nucleotides. A putative promoter sequence with -10 (TATATC) and -35 (TTGGCC) regions lies 50 nucleotides upstream of the start codon (ATG). The presumed initiator methionine is preceded by a

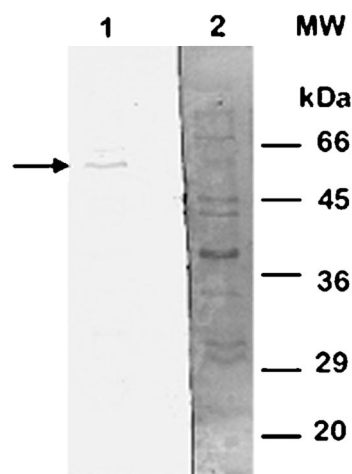


FIG. 1. Immunoblot analysis of *E. chrysanthemi* outer-membrane proteins. Lane 1, results of EC16 outer-membrane protein immunoblot experiments using anti-*E. coli* TolC rabbit antiserum; lane 2, Coomassie-stained EC16 outer-membrane proteins. The arrow indicates a band representing an outer-membrane polypeptide with an apparent mass of 51 kDa. MW, molecular mass standards.

canonical ribosome-binding site (AGGA) at six nucleotides upstream (36). The deduced amino acid sequence bears approximately 70% identity to that of the *E. coli* TolC. Multiple sequence alignment (CLUSTAL W [45]) of EC16 TolC with homologues from other bacteria (*E. coli*, *S. marcescens*, *S. enterica* serovar Enteritidis, and *V. cholerae*) revealed approximately 35% amino acid identity across all five sequences. The deduced amino acid sequence of the *E. chrysanthemi tolC* gene suggests that the protein possesses a signal peptide at its amino terminus. The 24-amino-acid sequence has the characteristics of a signal peptide (30) followed by a peptidase cleavage site between ala24 and glu25. This signal peptide is identical in sequence to the *E. coli* TolC signal peptide (17).

*E. chrysanthemi* carries an outer-membrane polypeptide with an apparent mass of 51 kDa that reacts with the anti-*E. coli* TolC rabbit antiserum (Fig. 1). This molecular mass was predicted from the deduced amino acid sequence and is slightly lower than that of the 52-kDa polypeptide observed in *E. coli*.

**Construction and characterization of the *E. chrysanthemi tolC* mutation.** Using a Kan resistance (*nptI*) cassette, the *tolC* gene of *E. chrysanthemi* EC16 was insertionally inactivated by marker-exchange mutagenesis (32). Confirmation of the chromosomal gene disruption was made by Southern blotting and PCR analysis using gene-specific probes and primers, respectively (data not shown). To characterize the phenotype of the EC16 *tolC* mutant (SF1603), we examined its sensitivity to a variety of agents that are substrates for *E. coli* MDR efflux systems: SDS, DOC, and NOV. The results are presented in Table 3 and indicate that the *tolC* mutation sensitizes this strain to all of these agents, suggesting that the role of TolC in resistance to antimicrobial and hydrophobic chemicals in *E. chrysanthemi* appears to be similar to that found in *E. coli*. Replacement of the *tolC* gene in *trans* on a plasmid (strain SF1603a) in SF1603 rescued the bacterium to drug resistance (Table 3). Strain SF1603 harboring the *E. coli tolC* gene on a plasmid (strain SF1603b) was also resistant to these antimicro-

TABLE 3. Sensitivity to antimicrobial agents<sup>a</sup>

Strain <sup>c</sup>	Diam (mm) of zone of inhibition <sup>b</sup> in the presence of:		
	NOV	SDS	DOC
EC16	20.00	16.33	6.00
SF1603	49.67	46.33	31.00
SF1603a	16.00	22.00	9.00
SF1603b	13.00	18.00	9.00

<sup>a</sup> Results represent averages from three separate experiments.  
<sup>b</sup> Disk diameter, 6 mm.  
<sup>c</sup> EC16, *E. chrysanthemi* EC16 parent strain; SF1603, EC16 *tolC* mutant strain; SF1603a, EC16 *tolC* mutant complemented with the functional EC16 *tolC* gene on plasmid pBR322; SF1603b, EC16 *tolC* mutant complemented with the functional *E. coli tolC* gene on plasmid pBR322.

bial chemicals. These results, together with those of the complementation studies performed using the cloned EC16 *tolC* in strain LM1151, suggest that the *tolC* genes from the two genera are interchangeable for drug resistance to certain antimicrobial chemicals. The MICs of SDS, DOC, and NOV for *E. chrysanthemi* EC16 (Table 4) are similar to those observed for *E. coli* (16). The MICs of these chemicals for the EC16 *tolC* mutant SF1603 were dramatically reduced and were approximately 500-fold lower than those for the wild type (Table 4).

**An *E. chrysanthemi tolC* mutant is defective in causing disease in plant tissue.** For many members of the *Enterobacteriaceae*, TolC has been implicated in a variety of functions (such as toxin secretion and MDR) that might play a role in pathogenicity. For *S. enterica* serovar Enteritidis, for example, TolC has been implicated in hemolysin secretion and virulence in BALB/c mice (40). In *E. coli*, hemolysin and enterotoxin secretion have been shown to require a functional TolC (27, 49); a *tolC* mutant of *V. cholerae* was found to be sensitive to bile salts, defective in cytotoxin secretion, and impaired in intestinal colonization (5). Since TolC is an important component in a variety of efflux pumps for the removal of many antimicrobial chemicals and in the secretion of certain virulence-associated proteins, we investigated the phytopathogenic potential of a *tolC* mutant of *E. chrysanthemi*.

The *E. chrysanthemi tolC* SF1603 mutant strain was compared with its wild-type parent in plant infections to assess the contribution of the protein to phytopathogenicity. Using witloof chicory leaves in a bioassay, we observed 95% (± 6.4%

TABLE 4. Minimum inhibitory concentration of antimicrobial agents<sup>a</sup>

Strain <sup>b</sup>	MIC (µg/ml) of:		
	NOV	SDS	DOC
EC16	1,000	>10,000 <sup>c</sup>	10,000
SF1603	<2 <sup>d</sup>	<20 <sup>e</sup>	39

<sup>a</sup> Average values of three separate experiments rounded off to nearest whole number are presented.  
<sup>b</sup> EC16, *E. chrysanthemi* EC16 wild type parent strain; SF1603, EC16 *tolC* mutant strain.  
<sup>c</sup> The wild-type parent strain grew in the presence of 10,000 µg of SDS/ml (highest concentration tested).  
<sup>d</sup> The *tolC* mutant strain did not grow in the presence of 2 µg of NOV/ml (lowest concentration tested).  
<sup>e</sup> The *tolC* mutant strain did not grow in the presence of 20 µg of SDS/ml (lowest concentration tested).

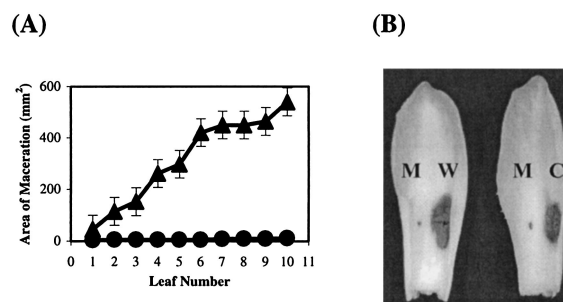


FIG. 2. *E. chrysanthemi* EC16 *tolC* mutant has severely reduced ability to produce lesions in witloof chicory leaves. (A) A total of 10 witloof chicory leaves were infected with either the *E. chrysanthemi* EC16 wild-type parent strain (closed triangles) or the *E. chrysanthemi* EC16 *tolC* mutant strain (closed circles). To estimate disease severity after incubation at 30°C for 72 h, the area of maceration was measured. Percent reduction in maceration was calculated as follows: [(area of maceration by wild-type strain – area of maceration by mutant strain)/ area of maceration by wild-type strain] × 100. The mean value (± standard deviation) of the percentage of reduction in maceration per 10 inoculations was used as a measure of the reduction in tissue maceration ability of the *tolC* mutant strain. (B) The *E. chrysanthemi tolC* mutant strain (M) was unable to macerate plant tissue but recovered almost wild-type (W) capacity to cause soft rot when the *tolC* gene was reintroduced into the mutant in *trans* (complemented strain) (C). Results representative of leaves after three independent experiments are shown. The relative positions (i.e., relative left-right orientations) of the inoculated strains did not have any effect on the extent of lesions produced (data not shown).

standard deviation) reduction in the area of tissue macerated with the *tolC* mutant (Fig. 2). This significant ( $P < 0.001$ ) reduction in phytopathogenic potential might reflect the inability of the bacterium to survive the toxic plant environment due to impairment of TolC-dependent chemical efflux pumps. Additionally, the mutation may impair secretion of virulence-associated proteins. However, no qualitative or quantitative differences were seen in the abilities of the *tolC* mutant strain and the wild-type parent strain to secrete proteases by using milk-derived proteins or gelatin as the substrate (11) or to secrete pectate lyases by using semisolid pectin agar and spectrophotometric assays (38) (data not shown).

**In planta survival and growth of the *E. chrysanthemi tolC* mutant.** Because the *E. chrysanthemi tolC* mutant was sensitive to many antimicrobial chemicals, we hypothesized that the bacterium would be unable to tolerate the toxic plant environment. We therefore compared the abilities of the *E. chrysanthemi tolC* mutant strain and the wild-type parent strain to survive and multiply in plant tissue (Fig. 3). The *E. chrysanthemi* wild-type strain multiplied in plant tissue, with bacterial numbers increasing approximately 10<sup>4</sup>-fold 78 h following inoculation. However, the total number of bacteria in the leaves inoculated with the *tolC* mutant was reduced by 40% 78 h following inoculation. The wild-type EC16 and the *tolC* mutant strains both multiplied to approximately 3.5 × 10<sup>7</sup> cells/gram of leaf tissue 6 h following inoculation, although no visible tissue maceration could be observed at this time. It was surprising that the *tolC* mutant was able to multiply in the plant tissue during the first 6 h. This could have been due to a lag time in the response of the leaves to infection.

Visible maceration of the plant tissue in the leaves infected

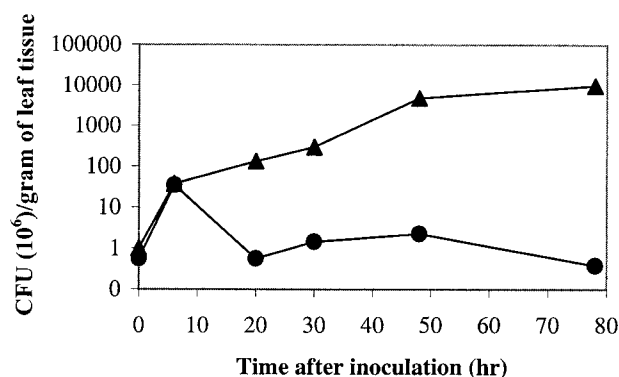


FIG. 3. Witloof chicory leaves inoculated with approximately  $7 \times 10^5$  bacteria of either the wild-type (closed triangles) or the *tolC* mutant (closed circles) strain were harvested at different time intervals to assess the number of viable bacteria. Results for number of CFU per gram of leaf tissue are plotted in a logarithmic scale along the y axis. A graph representative of two independent experiments is shown.

with wild-type EC16 strain was seen beginning just 12 h after initial inoculation. The total number of wild-type EC16 bacteria corresponding to the 20-h time interval was approximately  $1.3 \times 10^8$  cells/gram of leaf tissue. The bacteria multiplied exponentially, and the area of maceration grew correspondingly with time. In the case of the *tolC* mutant, after the initial increase in bacterial number the total number of bacteria decreased to the level of the inoculum after 20 h and to approximately 60% of the initial inoculum after 78 h. No visible tissue maceration was observed in the leaves inoculated with the *tolC* mutant at any time following inoculation. Hence, although the *tolC* strain survived, it was unable to grow and produce lesions in the leaf. This may have been due to the presence of some of the growth-inhibitory compounds produced by the leaves in response to infection. However, the amount of these compounds produced in the detached leaves (i.e., the ones used in this assay) apparently was not sufficient to kill all of the *tolC* mutant bacteria.

**The *E. chrysanthemi tolC* mutant is sensitive to antimicrobial plant chemicals.** Plants produce a variety of chemicals with antimicrobial properties (15, 42). Many bacteria have evolved mechanisms to tolerate and rid themselves of plant-derived toxins (43). Since the growth of the *E. chrysanthemi tolC* mutant was suppressed in plant tissue, we used a MIC assay and a variety of plant-derived antimicrobial chemicals to compare the sensitivities of the wild-type and mutant strains (Table 5). The *tolC* mutant clearly showed increased sensitivity to most of the plant chemicals we tested. When compared to those for the wild-type parent strain, the MICs of rhein, genistein, berberine, and plumbagin for the *tolC* mutant strain were found to be at least 64-fold, 32-fold, 16-fold, and 8-fold lower, respectively. Rhein is a well-characterized antimicrobial of rhubarb and is known to be effective against bacteria and *Saccharomyces cerevisiae* (12, 19). Genistein is an isoflavonoid produced by both legumes and nonleguminous plants (47, 50). It is a common precursor in the biosynthesis of antimicrobial phytoalexins and phytoanticipins and is also antibacterial (1, 3). Plumbagin is a naphthoquinone with known antimicrobial properties (13). The phenolic compounds *p*-coumaric acid and *t*-cinnamic acid

TABLE 5. MICs of model substrates and plant-derived chemicals<sup>a</sup>

Plant chemical	MIC ( $\mu\text{g/ml}$ ) of <sup>b</sup> :		
	WT	$\Delta\text{tolC}$	Several fold difference
Berberine	>2,000	125	>16
<i>t</i> -Cinnamic acid	500	250	2
<i>p</i> -Coumaric acid	500	125	4
Esculetin	500	125	4
Genistein	>1,000	31.25	32
Gossypol	>2,000	>2,000	
Plumbagin	31.25	3.91	8
Pyrrithione	3.13	1.56	2
Rhein	>1,000	15.63	64

<sup>a</sup> Results represent average values from three separate experiments.

<sup>b</sup> WT, *E. chrysanthemi* parent strain;  $\Delta\text{tolC}$ , *E. chrysanthemi tolC* mutant strain. Higher concentrations of some of the plant chemicals could not be tested due to limited solubility of these compounds.

as well as pyrithione and esculetin were slightly more toxic to the *tolC* mutant than to the wild-type parent. Pyrithione was seen to inhibit growth even of wild-type cells at concentrations as low as  $3.1 \mu\text{g/ml}$ . Wild-type strain EC16, as well as the *tolC* mutant strain, was resistant to relatively high levels (>2 mg/ml) of gossypol, a known antifungal phytoalexin of *Gossypium* species (8). This indicates that the *tolC* mutant is not nonspecifically sensitive to all chemicals. Berberine is an alkaloid with antibacterial properties. Interestingly, this compound has previously been observed to be toxic only to gram-positive bacteria. In gram-positive bacteria, the NorA pump has been implicated in resistance to this compound (39). Our data indicate that the loss of TolC sensitizes *E. chrysanthemi* to this compound because it accumulates inside the cells (Fig. 4).

Berberine has an increased fluorescence upon binding to DNA and thus represents a good model substrate for assessment of the efflux activity in the *tolC* mutant strain. The wild-type strain was found to be effective in keeping the intracellular concentrations of berberine low. However, the compound accumulated rapidly in the *tolC* mutant strain. These results suggest a role for TolC-mediated efflux of berberine in *E.*

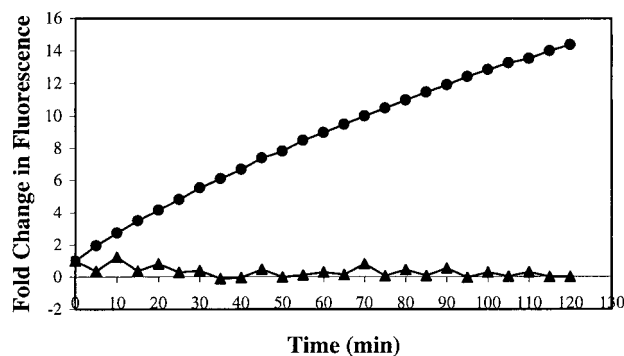


FIG. 4. Berberine accumulation was studied with the addition of 0.03 mg of berberine/ml to wild-type (closed triangles) or *tolC* mutant (closed circles) cells energized in the presence of 100 mM formate. Fluorescence was measured using a 360/40-nm excitation filter and a 530/25-nm emission filter. A graph representative of three independent experiments is shown above. Several-fold changes in fluorescence were calculated using average values of triplicates within a single experiment.

*chrysanthemii* and are compatible with those presented in Table 5.

In this study we have shown that *E. chrysanthemii* harbors a functional homologue of the *E. coli* TolC protein. The protein was localized to the outer membrane and found to be immunologically similar to *E. coli* TolC. The *E. chrysanthemii* TolC was able to replace its *E. coli* counterpart in chemical efflux. We also were able to demonstrate that a *tolC* mutant of *E. chrysanthemii* could not effectively infect witloof chicory leaves. The plant environment is replete with antimicrobial enzymes and chemicals, many of which are induced following infection by microorganisms. Thus, the inducible plant cellular defensive response functions to block pathogen amplification and impair ingress into plant tissue. Plant-derived secondary chemicals such as phytoalexins and hydrophobic chemicals like phenolics are recognized for their antimicrobial properties. Survival and sustained growth of successful plant pathogens in the plant necessitates the functioning of mechanisms to detoxify and/or expel plant-derived toxins. In *E. chrysanthemii* EC16, the *sapA-sapF* locus conferring resistance to antimicrobial peptides from plants has been shown to be important in survival of the bacterium in planta (28).

However, although much is understood about the virulence capabilities of many bacterial plant pathogens, little is known about the mechanisms employed by phytopathogens to survive the onslaught of the plant chemical environment. The *tolC* mutant of *E. chrysanthemii* was severely impaired in plant tissue maceration and in causing soft rot of witloof chicory. This mutation did not impair secretion of extracellular proteases or pectinases. This suggested that because of its role in antimicrobial chemical resistance, TolC is essential for bacterial survival in planta. We found that the *E. chrysanthemii tolC* mutant was unable to multiply effectively in plant tissue and was therefore unable to initiate soft rot and disease. This indicates that the infected leaf tissue was able to suppress the growth of the *tolC* mutant strain. The *E. chrysanthemii tolC* mutant strain was found to be sensitive to several plant chemicals, as has been observed with an *E. coli tolC* mutant strain (47). Analysis of one of the model plant chemicals, berberine, showed that the *tolC* mutant was defective in the extrusion of this compound. These data suggest that TolC-mediated MDR efflux may be vital to *E. chrysanthemii* during plant infection. We are presently examining the role of MDR efflux systems and their substrates in the phytopathogenicity of *E. chrysanthemii*.

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#### REFERENCES

- Akeda, Y., K. Nagayama, K. Yamamoto, and T. Honda. 1997. Invasive phenotype of *Vibrio parahaemolyticus*. *J. Infect. Dis.* **176**:822–824.
- Andersen, C., E. Koronakis, C. Hughes, and V. Koronakis. 2002. An aspartate ring in the TolC tunnel entrance determines ion selectivity and presents a target for blocking by large cations. *Mol. Microbiol.* **44**:1131–1139.
- Bae, E. A., M. J. Han, and D. H. Kim. 2001. In vitro anti-*Helicobacter pylori* activity of irisolidone isolated from the flowers and rhizomes of *Pueraria thunbergiana*. *Planta Med.* **67**:161–163.
- Barras, F., F. van Gijsegem, and A. K. Chatterjee. 1994. Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. *Annu. Rev. Phytopathol.* **32**:201–234.
- Bina, J. E., and J. J. Mekalanos. 2001. *Vibrio cholerae tolC* is required for bile resistance and colonization. *Infect. Immun.* **69**:4681–4685.
- Binet, R., and C. Wandersman. 1996. Cloning of the *Serratia marcescens hasF* gene encoding the Has ABC exporter outer membrane component: a TolC analogue. *Mol. Microbiol.* **22**:265–273.
- Borges-Walmsley, M. I., and A. R. Walmsley. 2001. The structure and function of drug pumps. *Trends Microbiol.* **9**:71–79.
- Bugeja, V., G. Charles, D. Collier, and D. Wilkie. 1988. Primary mitochondrial activity of gossypol in yeast and mammalian cells. *Biochem. Pharmacol.* **37**:4217–4224.
- Collmer, A. 1998. Determinants of pathogenicity and avirulence in plant pathogenic bacteria. *Curr. Opin. Plant Biol.* **1**:329–335.
- Collmer, A., and N. T. Keen. 1986. The role of pectic enzymes in plant pathogenesis. *Annu. Rev. Phytopathol.* **24**:383–453.
- Dahler, G. S., F. Barras, and N. T. Keen. 1990. Cloning of genes encoding extracellular metalloproteases from *Erwinia chrysanthemii* EC16. *J. Bacteriol.* **172**:5803–5815.
- Didry, N., L. Dubreuil, and M. Pinkas. 1994. Activity of anthraquinonic and naphthoquinonic compounds on oral bacteria. *Pharmazie* **49**:681–683.
- Didry, N., L. Dubreuil, F. Trotin, and M. Pinkas. 1998. Antimicrobial activity of aerial parts of *Drosera peltata* Smith on oral bacteria. *J. Ethnopharmacol.* **60**:91–96.
- Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski. 1980. Broad host range cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc. Natl. Acad. Sci. USA* **77**:7347–7351.
- Dixon, R. A. 2001. Natural products and plant disease resistance. *Nature* **411**:843–847.
- Fralick, J. 1996. Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. *J. Bacteriol.* **178**:5803–5805.
- Hackett, J., R. Misra, and P. Reeves. 1983. The TolC protein of *Escherichia coli* K12 is synthesized in a precursor form. *FEBS Lett.* **156**:307–310.
- Haseloff, B. J., T. L. Freeman, V. Valmeekam, M. W. Melkus, F. Oner, M. S. Valachovic, and M. J. D. San Francisco. 1998. The *exuT* gene of *Erwinia chrysanthemii* EC16: nucleotide sequence, expression, localization, and relevance of the gene product. *Mol. Plant-Microbe Interact.* **11**:270–276.
- Hatano, T., H. Uebayashi, H. Ito, S. Shiota, T. Tsuchiya, and T. Yoshida. 1999. Phenolic constituents of Cassia seeds and antibacterial effect of some naphthalenes and anthraquinones on methicillin-resistant *Staphylococcus aureus*. *Chem. Pharm. Bull.* **47**:1121–1127.
- Hugouvieux-Cotte-Pattat, N., and S. Reverchon. 2001. Two transporters, TogT and TogMNAB, are responsible for oligogalacturonide uptake in *Erwinia chrysanthemii* 3937. *Mol. Microbiol.* **41**:1125–1132.
- Hugouvieux-Cotte-Pattat, N., G. Condemine, W. Nasser, and S. Reverchon. 1996. Regulation of pectinolysis in *Erwinia chrysanthemii*. *Annu. Rev. Microbiol.* **50**:213–257.
- Hugouvieux-Cotte-Pattat, N., N. Blot, and S. Reverchon. 2001. Identification of TogMNAB, an ABC transporter which mediates the uptake of pectic oligomers in *Erwinia chrysanthemii* 3937. *Mol. Microbiol.* **41**:1113–1123.
- Johnson, O. L. 1998. The TolC homologue of *Erwinia chrysanthemii*. Ph.D. dissertation. Texas Tech University, Lubbock, Tex.
- Koronakis, V., A. Sharff, E. Koronakis, B. Luisi, and C. Hughes. 2000. Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* **405**:914–919.
- Letoffe, S., J. M. Ghigo, and C. Wandersman. 1994. Secretion of the *Serratia marcescens* HasA protein by an ABC transporter. *J. Bacteriol.* **176**:5372–5377.
- Letoffe, S., P. Delepelaire, and C. Wandersman. 1990. Protease secretion by *Erwinia chrysanthemii*: the specific functions are analogous to those of *Escherichia coli* alpha-hemolysin. *EMBO J.* **9**:1375–1382.
- Lewis, K. 1999. Multidrug resistance efflux, p. 15–40. In J. K. Broome-Smith, S. C. Baumberg, J. Stirling, and F. B. Ward (ed.), *Transport of molecules across microbial membranes*. Cambridge University Press, Cambridge, United Kingdom.
- Lopez-Solanilla, E., F. Garcia-Olmedo, and P. Rodriguez-Palenzuela. 1998. Inactivation of the *sapA* to *sapF* locus of *Erwinia chrysanthemii* reveals common features in plant and bacterial pathogenesis. *Plant Cell* **10**:917–924.
- Ma, D., D. N. Cook, M. Alberty, N. G. Pong, H. Nikaido, and J. E. Hearst. 1993. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. *J. Bacteriol.* **175**:6299–6313.
- Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* **10**:1–6.
- Otsuji, N., T. Soejima, S. Maki, and H. Shinagawa. 1982. Cloning of colicin E1 tolerant *tolC* (*mtcB*) gene of *Escherichia coli* K12 and identification of its gene product. *Mol. Gen. Genet.* **187**:30–36.
- Roeder, D. L., and A. Collmer. 1985. Marker exchange mutagenesis of pectate lyase isozyme gene in *Erwinia chrysanthemii*. *J. Bacteriol.* **164**:51–56.
- Salmond, G. P., and P. J. Reeves. 1993. Membrane traffic wardens and protein secretion in gram-negative bacteria. *Trends Biochem. Sci.* **18**:7–12.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

35. Shevchik, V. E., J. Robert-Baudouy, and G. Condemine. 1993. Specific interaction between OutD, an *Erwinia chrysanthemi* outer membrane protein of the general secretory pathway, and secreted proteins. *EMBO J.* **16**:3007–3016.
36. Shine, J., and L. Dalgarno. 1974. Identical 3'-terminal octanucleotide sequence in 18S ribosomal ribonucleic acid from different eukaryotes. A proposed role for this sequence in the recognition of terminator codons. *Biochem. J.* **141**:609–615.
37. Starr, M. P., and A. K. Chatterjee. 1972. The genus *Erwinia*: enterobacteria pathogenic to plants and animals. *Annu. Rev. Microbiol.* **26**:389–426.
38. Starr, M. P., A. K. Chatterjee, P. B. Starr, and G. E. Buchanan. 1977. Enzymatic degradation of polygalacturonic acid by *Yersinia* and *Klebsiella* species in relation to clinical laboratory procedures. *J. Clin. Microbiol.* **6**:379–386.
39. Stermitz, F. R., P. Lorenz, J. N. Tawara, L. A. Zenewicz, and K. Lewis. 2000. Synergy in a medicinal plant: antimicrobial action of berberine potentiated by a 5'-methoxyhydrnocarbin, a multidrug pump inhibitor. *Proc. Natl. Acad. Sci. USA* **97**:1433–1437.
40. Stone, B. J., and V. L. Miller. 1995. *Salmonella enteritidis* has a homolog of *tolC* that is required for virulence in BALB/c mice. *Mol. Microbiol.* **17**:701–712.
41. Sulavik, M. C., C. Houseweart, C. Cramer, N. Jiwani, N. Murgolo, J. Greene, B. DiDomenico, K. J. Shaw, G. H. Miller, R. Hare, and G. Shimer. 2001. Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. *Antimicrob. Agents Chemother.* **45**:1126–1136.
42. Swain, T. 1974. Biochemical evolution in plants, p. 125–302. *In* A. M. Florin and E. H. Stotz (ed.), *Comparative biochemistry, molecular evolution*, vol. 29, part I. Elsevier Scientific Publishing Company, New York, N.Y.
43. Tegos, G., F. R. Stermitz, O. Lomovskaya, and K. Lewis. 2002. Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. *Antimicrob. Agents Chemother.* **46**:3133–3141.
44. Thanassi, D. G., L. W. Cheng, and H. Nikaido. 1997. Active efflux of bile salts by *Escherichia coli*. *J. Bacteriol.* **179**:2512–2518.
45. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.
46. Van Gijsegem, F., and A. Toussaint. 1983. In vivo cloning of *Erwinia carotovora* genes involved in the catabolism of hexuronates. *J. Bacteriol.* **154**:1227–1235.
47. Van Rhijn, R., and J. Vanderleyden. 1995. The *Rhizobium*-plant symbiosis. *Microbiol. Rev.* **59**:124–142.
48. Wandersman, C., and P. Delepelaire. 1990. TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. *Proc. Natl. Acad. Sci. USA* **87**:4776–4780.
49. Yamanaka, H., T. Nomura, Y. Fujii, and K. Okamoto. 1998. Need for TolC, an *Escherichia coli* outer membrane protein, in the secretion of heat-stable enterotoxin I across the outer membrane. *Microb. Pathog.* **25**:111–120.
50. Yu, O., J. Woosuk, J. Shi, R. A. Croes, G. M. Fader, B. McGonigle, and J. T. Odell. 2000. Production of the isoflavones genistein and daidzein in non-legume and monocot tissues. *Plant Physiol.* **124**:781–793.