Tol-Pal proteins are critical cell envelope components of *Erwinia chrysanthemi* affecting cell morphology and virulence

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The *tol-pal* genes are necessary for maintaining the outer-membrane integrity of Gram-negative bacteria. These genes were first described in *Escherichia coli*, and more recently in several other species. They are involved in the pathogenesis of *E. coli*, *Haemophilus ducreyi*, *Vibrio cholerae* and *Salmonella enterica*. The role of the *tol-pal* genes in bacterial pathogenesis was investigated in the phytopathogenic enterobacterium *Erwinia chrysanthemi*, assuming that this organism might be a good model for such a study. The whole *Er. chrysanthemi* *tol-pal* region was characterized. Tol-Pal proteins, except TolA, showed high identity scores with their *E. coli* homologues. *Er. chrysanthemi* mutants were constructed by introducing a *uidA*–kan cassette in the *ybgC, tolQ, tolA, tolB*, and *pal* genes. All the mutants were hypersensitive to bile salts. Mutations in *tolQ, tolA*, *tolB* and *pal* were deleterious for the bacteria, which required high concentrations of sugars or osmoprotectants for their viability. Consistent with this observation, they were greatly impaired in their cell morphology and division, which was evidenced by observations of cell filaments, spherical forms, membrane blebbing and mislocalized bacterial septa. Moreover, *tol-pal* mutants showed a reduced virulence in a potato tuber model and on chicory leaves. This could be explained by a combination of impaired phenotypes in the *tol-pal* mutants, such as reduced growth and motility and a decreased production of pectate lyases, the major virulence factor of *Er. chrysanthemi*.

INTRODUCTION


The transcriptional organization of the *E. coli* *tol-pal* genes has been characterized. The genes *ybgC* (orf1), *tolQ, tolR, tolA* and *tolB*, and *pal* and *ybgF* (orf2) form two operons (Muller & Webster, 1997); a large *ybgC–ybgF* transcript has also been postulated (Vianney *et al.*, 1996). *ybgC* and *ybgF* encode proteins of unknown function located in the cytoplasm and the periplasm, respectively (Clavel *et al.*, 1996; Sun & Webster, 1987). Inactivation of these two ORFs induces no obvious phenotype in *E. coli*. In contrast, mutations in the *tol-pal* genes cause the disruption of outer-membrane integrity, which is evidenced by several phenotypes, including release of periplasmic content, sensitivity to bile salts and other chemical compounds, formation of outer-membrane blebs at the cell surface, and overproduction of colanic acid (Bernadac *et al.*, 1998; Clavel *et al.*, 1996; Vianney *et al.*, 1994). The *tol-pal* genes are also necessary for proper functioning of some uptake systems at the level of the cytoplasmic membrane (Llamas *et al.*, 2003a). In *E. coli*, group A colicins and filamentous-phage DNA use the Tol proteins for their translocation across the cell envelope.
(Bouveret et al., 1998; Webster, 1991). The existence of tol-pal has now been established in several Gram-negative bacteria (Bowe et al., 1998; Dennis et al., 1996; Heilpern & Waldor, 2000; Llamas et al., 2000; Prouty et al., 2002; Youderian et al., 2003). There is also evidence that some of the tol-pal genes are involved in the pathogenesis of E. coli (Hellman et al., 2002), Haemophilus ducreyi (Fortney et al., 2000), Salmonella enterica (Bowe et al., 1998) and Vibrio cholerae (Heilpern & Waldor, 2000). However, the way that the tol-pal genes are involved in the pathogenesis of these bacteria is not well documented, except in V. cholerae, where tolQRA mutants show defects in the uptake of ctxΦ DNA, which encodes cholera toxin (Heilpern & Waldor, 2000; Llamas et al., 2003). There is also evidence that some of the Erwinia chrysanthemi tol-pal genes may be a good alternative to study the involvement of the Tol-Pal proteins in pathogenicity. It is responsible for soft rot in many plants, including vegetable and ornamental species. It colonizes parenchymatous tissues by degrading the plant cell wall by means of a battery of pectinolytic enzymes. The oligosaccharides originating from pectin degradation are used as a carbon source by the bacterium (Hugouvieux-Cotte-Pattat et al., 2001).

Analysis of the role of the tol-pal genes in the pathogenesis of such phytopathogenic species may be easier than in animal pathogens and could reveal additional properties of the Tol-pal proteins that may be difficult to observe in the E. coli K-12 laboratory strains. In addition, complementation studies and the use of hybrid proteins between the two bacteria may help in the understanding of some unresolved features of the Tol-Pal system. In a first attempt, the Er. chrysanthemi tol-pal genes were cloned and sequenced, and mutants in most genes were isolated and characterized.

METHODS

Bacterial strains, plasmids and growth conditions. Er. chrysanthemi and E. coli derivatives are listed in Table 1. Plasmids used for subcloning experiments were pJEL250 (Valentin-Hansen et al., 1986), pBR328 (Soberon et al., 1980) and pKO3 (Link et al., 1997). Plasmid pN496 was a pBluescript derivative from the laboratory collection, containing a uidA–kan cassette flanked by multiple cloning sites.

Strains were grown in LB broth (with 5 g l⁻¹ NaCl) or M63 minimal medium at 37°C for E. coli or 30°C for Er. chrysanthemi (Miller, 1992). Tryptone swarm plates were used for motility assays and contained 3·5 g l⁻¹ Bacto agar (Difco), 10 g l⁻¹ Bacto tryptone (Difco) and 5 g l⁻¹ NaCl. The osmoprotectant glycine betaine was added to the growth medium at a final concentration of 1–10 mM. Antibiotics were added at a final concentration of 100 µg ml⁻¹ for ampicillin and 20 µg ml⁻¹ for kanamycin. Bacterial growth was monitored by OD₆₀₀.

Transfer of RP4 derivative plasmids by mating. pULB110, a kanamycin-sensitive derivative of RP4::mini-Mu, was used to generate R-prime derivatives containing bacterial DNA (van Gijsen et al., 1985). Mating between the recipient E. coli strain JC11305 and Er. chrysanthemi donor strains carrying plasmids was performed by spreading 0·2 ml of overnight cultures of the strains on M63 plates and incubating for 5 h at 30°C. Bacteria were resuspended in 1 ml of M63 medium and spread on selective media.

Cloning and sequencing of Er. chrysanthemi tol-pal genes. Most techniques were performed as described by Sambrook et al. (1989). For Southern blot analysis, DNA probes were prepared using the DIG High prime DNA labelling and detection starter kit (Roche). Recombinant plasmids were introduced into E. coli after a CaCl₂ treatment or into Er. chrysanthemi by electroporation at room temperature. Nucleotide sequencing was performed by Genome Express SA (Grenoble, France).

Construction of uidA–kan insertions and marker exchange recombination. The ybgC, tolQ, tolA, tolB, pal and ybgF genes were individually subcloned into pBR328 in order to generate plasmids with unique restriction sites in each gene. Two methods were used for marker exchange recombination. In the first one, the uidA–kan cassette of pN496 was introduced in the following unique sites: Msc

Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Properties</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli JC8056</td>
<td>supE44 hsdS metB1 gal lacY fluA ΔlacU169</td>
<td>Lab. collection</td>
</tr>
<tr>
<td>JC9776</td>
<td>8056 ΔybgCtolQRA</td>
<td>Lab. collection</td>
</tr>
<tr>
<td>JC11305</td>
<td>8056 uidA::Tn10 ΔtolBpal</td>
<td>Lab. collection</td>
</tr>
<tr>
<td>Er. chrysanthemi 3937</td>
<td>Wild-type</td>
<td>Lab. collection</td>
</tr>
<tr>
<td>MC12001</td>
<td>A3937 ybgC1::uidA–kan, insertion at MscI after aa 8 of YbgC</td>
<td>This work</td>
</tr>
<tr>
<td>MC12002</td>
<td>A3937 tolQ1::uidA–kan, insertion at HinDIII, removes the 27 C-terminal aa of TolQ</td>
<td>This work</td>
</tr>
<tr>
<td>MC12007</td>
<td>A3937 ybgF1::uidA–kan, insertion at EcoRV, after aa 69 of YbgF</td>
<td>This work</td>
</tr>
<tr>
<td>MC11634</td>
<td>A3937 tolA1::uidA–kan, insertion at HinDIII, removes the 7 C-terminal aa of TolA</td>
<td>This work</td>
</tr>
<tr>
<td>JF11970</td>
<td>A3937 tolQ2::uidA–kan, insertion at NheI, after aa 13 of TolQ</td>
<td>This work</td>
</tr>
<tr>
<td>JF11971</td>
<td>A3937 tolA2::uidA–kan, insertion at StuI, removes the 54 C-terminal aa of TolA</td>
<td>This work</td>
</tr>
<tr>
<td>JF11972</td>
<td>A3937 tolB1::uidA–kan, insertion at HpaI, removes the 59 C-terminal aa of TolB</td>
<td>This work</td>
</tr>
<tr>
<td>JF11973</td>
<td>A3937 pal1::uidA–kan, insertion at StuI, after aa 10 of the Pal signal peptide</td>
<td>This work</td>
</tr>
</tbody>
</table>
for ybgC, HindIII for tolQ and tolA, HpaI for tolB, SmaI for pal and EcoRV for ybgF (Fig. 1). In this cassette, the promoter region of uidA was absent, allowing the generation of a transcriptional fusion between the gene containing the insert and uidA. Plasmids containing the genes inactivated by the insertion were introduced into Er. chrysanthemi cells by electroporation. Integration of the insertions into the Er. chrysanthemi chromosome by marker exchange recombination was favoured by prolonged culture in low-phosphate medium.

Fig. 1. (A) Physical organization of the tol-pal locus of Er. chrysanthemi. The region cloned into pMC2256 corresponding to the AJ297885 sequence is shown (thick line, 6967 bp). The lines at the top represent the genomic DNA fragments carried by pMC2242 and pMC2244 used to obtain pMC2256. The location of the genes on the 6967 bp fragment is as follows: cydB, 1–367; ybgT, 384–500; ybgE, 497–790; ybgC, 925–1329; tolQ, 1326–2012; tolR, 2025–2450; tolA, 2579–3766; tolB, 3909–5195; pal, 5234–5740; ybgF, 5750–6574; lysT, 6741–6829. The potential rho-dependent terminator of transcription downstream from ybgF is indicated by a loop. (B) Predicted length of the different proteins and homology between Er. chrysanthemi and E. coli proteins. (C) Partial amino acid sequence of the TolA central domain of E. coli and Er. chrysanthemi, showing the three stretches that are lacking in Er. chrysanthemi. Homology between residues: identity (*), strong similarity (:), weak similarity (.).
in the presence of kanamycin (Roeder & Collmer, 1985). Colonies recovered on kanamycin LB plates were analysed by replica plating on ampicillin LB plates to confirm the loss of the plasmid vector (referred to as method 1). In the second method, the uidA–kan cassette was inserted in the Nhel, StuBI, Hpal and Stul sites of tolQ, tola, tolB and pal, respectively. Fragments overlapping the insertion were cloned into the Smal or NotI sites of pK03, a gene replacement vector that contains a temperature-sensitive origin of replication and markers for positive and negative selection for chromosomal integration and excision (Link et al., 1997). After electroporation, integration of the plasmid into the chromosome was selected by growth at 43 °C in the presence of kanamycin, then integmates were resolved at 30 °C and plasmid loss was selected in the presence of 10% sucrose. The colonies were then screened for sucrose resistance and the loss of chloramphenicol resistance by replica plating. For both protocols, the correct inactivation of the genes was controlled by PCR amplification of the chromosomal DNA, using the first primer in the uidA gene and the second primer in the tol gene. A control was also carried out to check for the absence of an intact gene.

**Motility assays.** Tryptone swarm plates were inoculated with a toothpick and bacteria were grown from 16 to 24 h at 30 °C. The diameter of the growth area for each mutant was compared to that of the wild-type strain. Plates were inoculated with a maximum of four bacterial strains per plate, including the wild-type as a control.

**Phenotype analysis of mutants.** Sensitivity towards antimicrobial agents was tested on LB plates by replica plating the strains on rich media containing various amounts of the tested agent. To test strain virulence, 105 cells were inoculated in chicory leaves after scarification. After 24 h of incubation at 30 °C, the length of the rotted region was measured. Potato tubers were inoculated as previously described (Lojkowska et al., 1995). A hole was made into the tuber parenchyma and 105 cells were used for inoculation. After 24–72 h incubation, tubers were sliced vertically through the infection point, and the weight of decayed tissue was taken as a measure of disease severity. All the macerated tissue was collected and used for bacterial counting. For each bacterium, 500 mg of macerated tissue was resuspended in 500 µl M63 medium, treated with 50 µl tolueol, and vortexed thoroughly. After centrifugation (12 000 g for 2 min), the supernatant was used to determine β-galactosidase (which reflected the colony number, see Methods, Enzyme assays, below) and pectate lyase activities.

**Microscopy.** Bacterial cells were directly observed after growth in LB medium, LB medium supplemented with 10% sucrose or in chicory leaves. This allowed us to estimate the morphology of the cells. Bacteria were also recorded using a camera to estimate their motility.

For transmission electron microscopy, cells were grown on LB plates or LB plates supplemented with 10% sucrose, recovered with a loop on a Parafilm sheet, and then fixed with osmium tetroxide vapours. They were resuspended in 0.1 M sodium cacodylate, and then stained with 1% sodium silicotungstate or uranyl acetate.

**Enzyme assays.** Pectate lyase activity was measured in 0-1 M Tris, pH 8.5, 0.1 mM CaCl2, 0.05% polygalacturionate, by following an increase in A238 from the cleavage of polygalacturonate. The assay for β-galactosidase activity has been described elsewhere (Miller, 1992). Because the cell morphology of the tol-pal mutants was heterogeneous, the OD600 did not necessarily reflect the amount of bacteria. Thus, we calculated the relative natural β-galactosidase activity of Er. chrysanthemi based on colony number, which was determined by cell enumeration. Accordingly, the pectate lyase activity of each strain was divided by its β-galactosidase activity to standardize the enzyme activity (referred to as ‘relative units’ in the text). Cell enumeration was determined by plate count.

**Western blot analyses.** Cells (3 × 108) in the mid-exponential phase of growth were centrifuged, resuspended in loading buffer and boiled. Samples were separated by SDS-PAGE [12% polyacrylamide, (Laemmli, 1970)] and transferred for 2 h onto a nitrocellulose membrane by using a semi-dry blotter. Immunoblots were developed with the BM chemiluminescence blotting substrate (Roche). Polyclonal antibodies raised against E. coli TolA, TolB, Pal and YbgF proteins have been previously described (Clavel et al., 1998) and were used to detect the corresponding Er. chrysanthemi proteins.

**Nucleotide sequence accession number.** The nucleotide sequence reported here, corresponding to a 6967 bp fragment, has been deposited at EMBL under the accession number AJ297885 (EMBL/genebank version AJ297885.1 GI:16116629).

**RESULTS**

**Complementation of the ΔtolBpal mutation of E. coli with an RP4 derivative of Er. chrysanthemi A3937**

We used the selectable marker nadA, located 3 kb downstream from the tol-pal locus of E. coli, to clone the tol-pal region of Er. chrysanthemi, since the genetic organization of several genomic regions is quite similar in E. coli and Er. chrysanthemi. Plasmid pULB110 was used to generate R-prime derivatives containing an insert of bacterial DNA from Er. chrysanthemi that complemented the nadA mutation of strain JC11305 (nadA::Tn10ΔtolBpal). Nad+ transconjugants were isolated on M63 medium plates without nicotinic acid. Plasmid pR’16 was retained for further studies, since it complemented not only the nadA mutation but also the cholate-sensitivity phenotype associated with the ΔtolBpal mutation. Transfer of pR’16 in JC9776 demonstrated that this plasmid also complemented the cholate-sensitivity phenotype of the ΔybgCtolQRAPA mutation.

**Cloning and sequencing of Er. chrysanthemi tol-pal genes**

Plasmid pMC2242, containing an 8·5 kb EcoRI fragment of pR’16 cloned into pJEL250, complemented the cholate-sensitivity phenotype of the ΔtolBpal mutation of JC11305. DNA sequence analysis revealed that it contained the ‘ybgCtolQRAPAlybgF’ region (Fig. 1). To clone the region upstream of ybgC, an EcoRI–StuBI fragment from pMC2242 containing the ‘ybgCtolQRAPA’ genes was used as a DNA probe for Southern analysis of pR’16 digested with HindIII. A 4·3 kb HindIII fragment was isolated and cloned into pBR322 to give pMC2244 (Fig. 1). Finally, a BamHI–Nhel fragment from pMC2244 was introduced into pMC2242 to give pMC2256. Further sequencing showed that it contained the entire ybgC sequence and its upstream region. The 6967 bp fragment cloned into pMC2256 is presented in Fig. 1(A). Upstream of ybgC, homologues of genes cydB (truncated), ybgT and ybgE of E. coli were identified. The region downstream from ybgF showed high similarity to lysT, indicating that the gene order in the tol-pal region of Er. chrysanthemi is the same as that found in E. coli. Unlike in E. coli, no potential transcription terminator could be
identified in the ybgE–ybgC intergenic region. A potential rho-dependent terminator is situated downstream from ybgF \( [\Delta G = -30.4 \text{ kcal mol}^{-1} (127 \text{ kJ mol}^{-1})] \), a characteristic also found in Pseudomonas putida and E. coli (Llamas et al., 2003b; Vianney et al., 1996). The Tol-Pal proteins of Er. chrysanthemi and E. coli displayed a high degree of similarity (Fig. 1B), except for TolA, which was the least-conserved protein. This is mainly due to its central domain, in which three stretches of seven amino acids are lacking in Er. chrysanthemi (Fig. 1C). This region is organized in an \( \alpha \)-helical structure that crosses the periplasmic space (Derouiche et al., 1999; Levengood et al., 1991).

**Gene inactivation**

The first method used for gene inactivation allowed us to obtain mutants ybgC1, tolQ1, tolA1 and ybgF1, containing a \( \textit{uidA} \)–kan cassette in the MscI, HindIII (for \( \textit{tolQ1} \) and \( \textit{tolA1} \)), and EcoRV sites, respectively. The correct insertion and orientation of the cassette in the chromosome were confirmed by PCR. In the course of our experiments, we observed that the \( \textit{tolQ1} \) mutation was highly unstable; this mutation could not be retained. Moreover, attempts to inactivate the chromosomal alleles of the \( \textit{tolB} \) and \( \textit{pal} \) genes using this method were unsuccessful. We hypothesized that prolonged culturing in low-phosphate medium could be lethal for some of the \( \textit{tol-pal} \) mutants. Hence, we used the second method (see Methods), which allowed growth in rich medium. Er. chrysanthemi was transformed with pK03 derivatives containing \( \textit{tolQ} \), \( \textit{tolA} \), \( \textit{tolB} \) and \( \textit{pal} \) inactivated by the \( \textit{uidA} \)–kan cassette in the NheI, SnaBI, Hpal and Stul sites, respectively (Fig. 1A). To our surprise, the colonies selected on LB plates supplemented with 10% sucrose grew very slowly on LB plates without sucrose. Therefore, we retained colonies which were able to grow on plates supplemented with sucrose and kanamycin but unable to grow on rich media supplemented with sucrose and chloramphenicol (loss of the pK03 vector), or cholate (\( \textit{tol} \) phenotype). Using this technique, we were able to obtain the mutants \( \textit{tolQ2} \), \( \textit{tolA2} \), \( \textit{tolB1} \) and \( \textit{pal1} \) (Table 1). The orientation of the \( \textit{uidA} \)–kan cassette was investigated and shown to generate a transcriptional fusion between the inactivated gene and \( \textit{uidA} \) in all but the \( \textit{tolA2} \) mutant. The poor growth of the \( \textit{tolB} \) and \( \textit{pal} \) mutants in the absence of sucrose provides a good explanation for our inability to isolate these mutants using the first method. The \( \textit{tolA1} \) mutant first isolated lacked only the seven C-terminal amino acid residues of TolA1, and comparison of the \( \textit{tolA1} \) and \( \textit{tolA2} \) mutants suggested that \( \textit{tolA1} \) retained a partial functionality. This could be due to the presence of higher amounts of TolB and Pal in the \( \textit{tolA1} \) mutant (Fig. 2). The instability of the \( \textit{tolQ1} \) mutant could also be explained by its poor ability to grow on LB plates.

**Analysis of Er. chrysanthemi TolA, TolB, Pal and YbgF synthesis by Western blotting**

The impact of the mutations on the production of the TolA, TolB, Pal and YbgF proteins was determined by immunodetection of the proteins. Cells were grown in 10% sucrose LB broth until mid-exponential phase. After separation by SDS-PAGE, Western blot analysis was carried out and polyclonal antibodies against the four proteins were used for immunodetection (Fig. 2). TolA, TolB, Pal and YbgF were missing in the \( \textit{tolA} \), \( \textit{tolB} \), \( \textit{pal} \) and \( \textit{ybgF} \) mutants, respectively. Many mutations led to the absence or a great reduction in the level of proteins encoded by downstream genes. For instance, both TolA and TolB levels were reduced in the \( \textit{ybgC} \), \( \textit{tolQ} \) and \( \textit{tolA} \) mutants. Pal was also reduced in \( \textit{tolA2} \) and \( \textit{tolB1} \) mutants. YbgF was reduced in most mutants, but particularly in the \( \textit{pal1} \) mutant. These results support the hypothesis of a large \( \textit{ybgC–ybgF} \) transcriptional unit that could lead to a polar effect of the upstream mutations. However, since many Tol-Pal proteins are known to form complexes, the instability of some of them may also result from the lack of their interacting partner(s).

**Er. chrysanthemi tol-pal mutants are impaired in cell motility and sensitive to ionic strength and osmolarity**

The wild-type strain, as well as the \( \textit{ybgC1} \), \( \textit{tolA1} \) or \( \textit{ybgF1} \) mutants, was able to grow on LB and M63 plates. In contrast, the \( \textit{tolQ2} \), \( \textit{tolA2} \), \( \textit{tolB1} \) and \( \textit{pal1} \) mutants did not form colonies on LB or M63 plates, except in the presence of 10% sucrose. They also grew very slowly in LB liquid medium and when they were patched on LB plates. The role of sucrose was unexpected. Its addition affects the medium osmolarity, but it can also act as a membrane and protein stabilizer in adverse conditions (Crowe et al., 1988; Leslie et al., 1995; Molina-Hoppner et al., 2004). To discriminate between these two effects, the mutants were grown on LB plates supplemented with sucrose, various amounts of sugars, NaCl or the osmoprotectant glycine betaine. The
Table 2. Motility of the *Er. chrysanthemi* tol-pal mutants

Swarm plates (with or without sucrose) were inoculated as described in Methods and grown at 30°C for 24 h. Results are the mean of three independent experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Swarm diameter (mm) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No sucrose</td>
</tr>
<tr>
<td>3937</td>
<td>43·0 ± 3·4</td>
</tr>
<tr>
<td>ybgC1</td>
<td>21·6 ± 3·6</td>
</tr>
<tr>
<td>tolQ2</td>
<td>8·0 ± 2·3</td>
</tr>
<tr>
<td>tolA1</td>
<td>7·6 ± 1·1</td>
</tr>
<tr>
<td>tolA2</td>
<td>10·0 ± 2·8</td>
</tr>
<tr>
<td>tolB1</td>
<td>0</td>
</tr>
<tr>
<td>pal1</td>
<td>6</td>
</tr>
<tr>
<td>ybgF1</td>
<td>36·8 ± 2·9</td>
</tr>
</tbody>
</table>

tolQ2, tolA2, tolB1 and pal1 mutants recovered a normal growth when sucrose was replaced by 10% glucose, galactose (two sugars metabolized by *Er. chrysanthemi*), maltose or lactose (two sugars not metabolized by *Er. chrysanthemi*), but not glycerol. Addition of the osmoprotective glycine betaine (1–10 mM) also allowed growth of the mutants.

When cells were recovered from LB plates supplemented with 10% sucrose, they were all surrounded by a capsule (data not shown). The altered morphologies identified after growth on LB plates were conserved, except that (i) the tolQ2 mutant formed short curved filaments with round poles, (ii) the tolA2 mutants formed filaments with cells of more homogeneous size and (iii) the tolB1 mutant did not have filaments, but rather formed swollen cells that still lacked flagella. Vesicles appeared at the cell surface as well as very large envelope excrescences.

Sensitivity to antimicrobial agents

The sensitivity of the mutants towards various antimicrobial agents was analysed (Table 3). All the mutants were more sensitive to sodium cholate than the wild-type strain. In *E. coli*, only tolQRAB pal mutants show this phenotype (Vianney et al., 1996). The sensitivity of the ybgC1 mutant to sodium cholate can be explained by the polarity of the insertion. Consistent with this hypothesis, this phenotype could be complemented by the addition of a multicopy plasmid carrying the *Er. chrysanthemi* ybgCtolQRA cluster, but not when the plasmid contained only ybgC (data not shown). The ybgF1 mutant was sensitive to cholate, but resistant to the other chemical compounds tested. This phenotype could be complemented by providing the tolBpalybgF genes in trans (data not shown). The other mutants were sensitive to SDS and carbonyl cyanide m-chlorophenylhydrazine (CCCP), but resistant to vancomycin (data not shown). The tolB1 and pal1 mutants were more sensitive to chemical compounds than the tolQ and tolA mutants. However, with the exception of SDS, most differences in sensitivity were moderate.

Complementation between *Er. chrysanthemi* and *E. coli*

As shown previously, the tol-pal genes of *Er. chrysanthemi* were able to complement the cholate sensitivity and the motility of the *E. coli* tol-pal mutants. In contrast, the *E. coli* tol-pal genes did not complement these genes when they were introduced in the *Er. chrysanthemi* tol-pal mutants, even if expression of the *E. coli* TolA, TolB and Pal proteins could be detected by Western blotting in the *Er.
chrysanthemi tolA, tolB and pal mutants. These data suggest either that the Er. chrysanthemi Tol-Pal proteins are involved in additional functions or that the E. coli Tol-Pal proteins are unable to interact appropriately with other components of the Er. chrysanthemi cell envelope.

**Virulence and survival in plant tissues of the ybgC, tolQ, tolA, tolB, pal and ybgF mutants**

The tolQ, tolA, tolB and pal mutants were strongly affected for virulence, since inoculation with these strains led to poor tissue maceration on chicory leaves (Fig. 4) or potato tubers (data not shown). Global pectate lyase activity (relative units) was strongly decreased in the macerated tissues of all the tol-pal mutants (ranging from 0.0008 to 0.008, while the activity of the wild-type strain was 0.02), decreased by two-fold in the ybgC1 mutant, but increased to 0.03 in the ybgF1 strain (data not shown). Wild-type and mutant strains were recovered from macerated tissue and observed under a phase-contrast microscope: their motility and morphology was analogous to that observed when the same strains were grown on 10% sucrose LB plates (data not shown).

**DISCUSSION**

This study was undertaken to extend information about the role of the ybgC tolQRAB pal ybgF genes in a...
phytopathogenic Gram-negative bacterium. Indeed, tolQRAB pal mutations have been extensively characterized in many Gram-negative bacteria (Dennis et al., 1996; Fortney et al., 2000; Heilpern & Waldor, 2000; Llamas et al., 2000; Prouty et al., 2002). Mutants in all these genes are impaired in the so-called phenotype of outer-membrane integrity: release of periplasmic content in the extracellular medium, increased sensitivity to deleterious agents such as bile salts, motility and outer-membrane blebbing (P. putida), and entry of filamentous phage DNA (E. coli, V. cholerae).

In Er. chrysanthemi, inactivation of tolQ, tolA, tolB or pal is deleterious, since mutants in these genes grow very slowly on LB plates. They can grow well only when the medium is supplemented with 10% sugars (sucrose, glucose, maltose or lactose) or with the osmoprotectant glycine betaine, but not in the presence of glycerol or 0.1–0.3 M NaCl. Glycine betaine and sugars are non-ionic compatible solutes that can be accumulated by de novo synthesis or transport without interfering with vital cellular processes. These compounds not only confer protection against high osmolarity, but also allow protein protection (Poolman & Glaasker, 1998). The addition of sugars or betaine to the growth medium probably contributes to the osmoprotection of some envelope components. Consistent with this hypothesis, all the tol-pal mutants showed increased resistance to chemical compounds in the presence of sucrose (data not shown), and the tolQ and tolA mutants were more motile under such conditions (Table 2). The addition of sugars may also contribute to the maintenance of a turgor pressure compatible with cell viability for the Er. chrysanthemi tol-pal mutants. As in P. putida (Llamas et al., 2000), we observed that the tolB and pal mutations led to more severe phenotypes than those observed with tolQ and tolA mutants. Attempts to construct tol-pal mutants in some bacteria have been unsuccessful, and could be explained by their poor growth in classical rich media, as observed for Er. chrysanthemi tol-pal mutants (Dennis et al., 1996; Spinola et al., 1996).

Our electron microscopy observations provide a good explanation for the impairment of cell motility of the tol-pal mutants. The tolB and pal mutants lack flagella, while tolQ and tolA mutants have fewer and shorter flagella, a phenotype which suggests an alteration of flagella synthesis, polymerization and/or stability.

The Er. chrysanthemi tol-pal mutants have an altered cell morphology: some of the cells do not correctly localize the septum and the poles during division. A phenotype of filamentation: some of the cells do not correctly localize the septum and the poles during division. A phenotype of filamentation has also been observed in tol-pal mutants of P. putida (Llamas et al., 2000), E. coli (Meury & Devilliers, 1999) and V. cholerae (Heilpern & Waldor, 2000), and the incorrect positioning of the septa has also been reported (Meury & Devilliers, 1999).

### Table 3. Sensitivity of Er. chrysanthemi tol-pal mutants to chemical compounds

Colonies were grown on LB plates supplemented by sodium cholate, SDS or CCCP. Growth was estimated after 24 h at 30°C. The maximal concentration of the chemical compounds allowing growth of the bacteria is given (mean of three experiments).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sodium cholate (mg ml(^{-1}))</th>
<th>SDS (mg ml(^{-1}))</th>
<th>CCCP (μg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>3937</td>
<td>15</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>ybgC1</td>
<td>10</td>
<td>0.9</td>
<td>20</td>
</tr>
<tr>
<td>tolQ2</td>
<td>7.5</td>
<td>0.5</td>
<td>15</td>
</tr>
<tr>
<td>tolA1</td>
<td>7.5</td>
<td>0.5</td>
<td>15</td>
</tr>
<tr>
<td>tolA2</td>
<td>7.5</td>
<td>0.5</td>
<td>15</td>
</tr>
<tr>
<td>tolB1</td>
<td>7.5</td>
<td>0.2</td>
<td>10</td>
</tr>
<tr>
<td>pal1</td>
<td>7.5</td>
<td>0.2</td>
<td>10</td>
</tr>
<tr>
<td>ybgF1</td>
<td>10</td>
<td>3</td>
<td>20</td>
</tr>
</tbody>
</table>

Fig. 4. Pathogenicity of tol-pal mutant strains on chicory leaves. Bacteria \((10^7)\) were inoculated into scarified chicory leaves. Disease symptoms were observed after 24 h. T, scarified but not inoculated leaf.
Erwinia chrysanthemi tol-pal mutants showed a reduced virulence on chicory leaves and potato tubers. Altered virulence of tolB and pal mutants has been reported in animal pathogen. In H. ducreyi, expression of Pal is required for virulence in a human model (Fortney et al., 2000). In E. coli, Pal is involved in Gram-negative sepsis (Hellman et al., 2002; Liang et al., 2005). In S. enterica, tolB mutants are attenuated in a mouse typhoid model of infection (Bowe et al., 1998). Although tolB mutants cross the gut, they are unable to cause fatal infection. This has been attributed to their inability to survive within macrophages and resist the bactericidal effects of non-immune serum. In the same screening, the authors also identified the mdoB gene as essential for fatal infection (Bowe et al., 1998). opg (or mdo) mutants have been associated with a lack of virulence in pathogen. The presence of Tol-Pal lipoprotein of E. coli tol-pal mutants may be involved in cell division (Carballes et al., 1997). Unlike the tol-pal mutants, the opgGH mutants show a complete loss of virulence in potato tubers, but present some phenotypes similar to those observed for the tol-pal mutants, namely reduced motility and pectate lyase production (Page et al., 2001). Another common trait between Opg and Tol-Pal is the activation of regulatory networks, such as the Rcs phosphorelay in the interaction with TolA and TolB proteins upon import into Escherichia coli. The TolQ-TolR two-component system rcsB plays a major role in Salmonella typhimurium genome is required for fatal infection of mice. Infect Immun 66, 3372–3377.

REFERENCES


Clavel, T., Germon, P., Vianney, A., Portailier, R. & Lazzaroni, J. C. (1998). TolB protein of Escherichia coli K-12 interacts with the outer membrane lipoprotein YbgF in the Tol-Pal system. This work further demonstrates that the Tol-Pal proteins are critical cell envelope components necessary for bacterial virulence. The low growth rate of the Erwinia chrysanthemi tol-pal mutants, their impaired viability, and their reduced motility and pectinase production are probably sufficient to explain the reduced virulence. It also provides further evidence that the loss of Tol-Pal functions can be deleterious for some bacterial species.

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membrane peptidoglycan-associated proteins Pal, Lpp and OmpA.


