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*, *pal* and *ybgF* 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*.

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INTRODUCTION

The Tol-Pal system of Gram-negative bacteria is required for outer-membrane stability (Lazzaroni *et al.*, 1999). It comprises five envelope proteins, TolQ, TolR, TolA, TolB and Pal, which form two complexes. The TolQ, TolR and TolA inner-membrane proteins interact via their trans-membrane domains (Derouiche *et al.*, 1995; Lazzaroni *et al.*, 1995). The β -propeller domain of the periplasmic protein TolB is responsible for its interaction with Pal (Ray *et al.*, 2000). TolB also interacts with the outer-membrane peptidoglycan-associated proteins Lpp and OmpA (Cascales *et al.*, 2002; Clavel *et al.*, 1998). TolA undergoes a conformational change in response to changes in the proton-motive force (Germon *et al.*, 2001), and interacts with Pal in an energy-dependent manner (Cascales *et al.*, 2001). The C-terminal periplasmic domain of TolA also interacts with the

N-terminal domain of TolB (Dubuisson *et al.*, 2002; Walburger *et al.*, 2002).

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

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence reported in this paper, corresponding to a 6967 bp fragment, is AJ297885.

(Bouveret *et al.*, 1998; Webster, 1991). The existence of *tol-pal* genes 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), and in *E. coli*, where Pal initiates inflammation in sepsis, in synergy with lipopolysaccharide (Liang *et al.*, 2005). The enterobacterium *Erwinia chrysanthemi* 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 Gijsegem *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: *MscI*

Table 1. Bacterial strains

aa, amino acid residue.

Strain	Properties	Source
<i>E. coli</i>		
JC8056	<i>supE44 hsdS metB1 gal lacY fhuA ΔlacU169</i>	Lab. collection
JC9776	8056 <i>ΔybgCtolQRA</i>	Lab. collection
JC11305	8056 <i>nad::Tn10 ΔtolBpal</i>	Lab. collection
<i>Er. chrysanthemi</i>		
3937	Wild-type	Lab. collection
MC12001	A3937 <i>ybgC1::uidA</i> -kan, insertion at <i>MscI</i> after aa 8 of YbgC	This work
MC12002	A3937 <i>tolQ1::uidA</i> -kan, insertion at <i>HindIII</i> , removes the 27 C-terminal aa of TolQ	This work
MC12007	A3937 <i>ybgF1::uidA</i> -kan, insertion at <i>EcoRV</i> , after aa 69 of YbgF	This work
MC11634	A3937 <i>tolA1::uidA</i> -kan, insertion at <i>HindIII</i> , removes the 7 C-terminal aa of TolA	This work
JF11970	A3937 <i>tolQ2::uidA</i> -kan, insertion at <i>NheI</i> , after aa 13 of TolQ	This work
JF11971	A3937 <i>tolA2::uidA</i> -kan, insertion at <i>SnaBI</i> , removes the 54 C-terminal aa of TolA	This work
JF11972	A3937 <i>tolB1::uidA</i> -kan, insertion at <i>HpaI</i> , removes the 59 C-terminal aa of TolB	This work
JF11973	A3937 <i>pal1::uidA</i> -kan, insertion at <i>StuI</i> , after aa 10 of the Pal signal peptide	This work

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 (.)

for *ybgC*, *HindIII* for *tolQ* and *tolA*, *HpaI* for *tolB*, *StuI* 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

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 *NheI*, *SnaBI*, *HpaI* and *StuI* sites of *tolQ*, *tolA*, *tolB* and *pal*, respectively. Fragments overlapping the insertion were cloned into the *SmaI* or *NotI* sites of pKO3, 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 integrates 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, 10⁷ 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 10⁷ 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 toluene, 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 tetrachloride 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 % polygalacturonate, by following an increase in A₂₃₀ 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/genbank 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*:::Tn10 Δ tolBpal). 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 $\Delta ybgCtolQRA$ 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 '*ybgCtolQRA*' region (Fig. 1). To clone the region upstream of *ybgC*, an *EcoRI*–*SnaBI* fragment from pMC2242 containing the '*ybgCtolQRA*' 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 pBR328 to give pMC2244 (Fig. 1). Finally, a *BamHI*–*NheI* 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 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 α -helical structure that crosses the periplasmic space (Derouiche *et al.*, 1999; Levenood *et al.*, 1991).

Gene inactivation

The first method used for gene inactivation allowed us to obtain mutants *ybgC1*, *tolQ1*, *tolA1* and *ybgF1*, containing a *uidA*-kan cassette in the *MscI*, *HindIII* (for *tolQ1* and *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 *tolQ1* mutation was highly unstable; this mutation could not be retained. Moreover, attempts to inactivate the chromosomal alleles of the *tolB* and *pal* genes using this method were unsuccessful. We hypothesized that prolonged culturing in low-phosphate medium could be lethal for some of the *tol-pal* mutants. Hence, we used the second method (see Methods), which allowed growth in rich medium. *Er. chrysanthemi* was transformed with pKO3 derivatives containing *tolQ*, *tolA*, *tolB* and *pal* inactivated by the *uidA*-kan cassette in the *NheI*, *SnaBI*, *HpaI* and *StuI* 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 pKO3 vector), or cholate (*tol* phenotype). Using this technique, we were able to obtain the mutants *tolQ2*, *tolA2*, *tolB1* and *pal1* (Table 1). The orientation of the *uidA*-kan cassette was investigated and shown to generate a transcriptional fusion between the inactivated gene and *uidA* in all but the *tolA2* mutant. The poor growth of the *tolB* and *pal* mutants in the absence of sucrose provides a good explanation for our inability to isolate these mutants using the first method. The *tolA1* mutant first isolated lacked only the seven C-terminal amino acid residues of TolA1, and comparison of the *tolA1* and *tolA2* mutants suggested that *tolA1* retained a partial functionality. This could be due to the presence of higher amounts of TolB and Pal in the *tolA1* mutant (Fig. 2). The instability of the *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

Fig. 2. Immunoblots of TolA, TolB, Pal and YbgF in whole-cell lysates of *Er. chrysanthemi* wild-type and mutant strains. Cells (3×10^8) were loaded on the gel. Proteins were separated by SDS-PAGE (12%, w/v), transferred and immunodetected as described in Methods.

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 *tolA*, *tolB*, *pal* and *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 *ybgC*, *tolQ* and *tolA* mutants. Pal was also reduced in *tolA2* and *tolB1* mutants. YbgF was reduced in most mutants, but particularly in the *pal1* mutant. These results support the hypothesis of a large *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 *ybgC1*, *tolA1* or *ybgF1* mutants, was able to grow on LB and M63 plates. In contrast, the *tolQ2*, *tolA2*, *tolB1* and *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.

Strain	Swarm diameter (mm) with:	
	No sucrose	Sucrose (10 %)
3937	43.0 ± 3.4	40.6 ± 3.4
<i>ybgC1</i>	21.6 ± 3.6	34.6 ± 3.9
<i>tolQ2</i>	8.0 ± 2.3	19.4 ± 4.8
<i>tolA1</i>	7.6 ± 1.1	13.4 ± 1.1
<i>tolA2</i>	10.0 ± 2.8	17.4 ± 2.3
<i>tolB1</i>	0	3.4 ± 0.5
<i>pal1</i>	0	5.0 ± 1.0
<i>ybgF1</i>	36.8 ± 2.9	38.2 ± 2.2

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 osmoprotectant glycine betaine (1–10 mM) also allowed growth of the mutants. In contrast, the *tolQ2*, *tolA2*, *tolB1* and *pal1* mutants did not grow when NaCl was added to LB plates at concentrations between 0.1 and 0.3 M. The presence of NaCl in the medium affects both osmolarity and ionic strength. Therefore, we concluded that the mutants were sensitive to high ionic strength and low osmolarity.

The motility of the mutants was tested on swarm plates, as described in Methods. After 20 h growth at 30 °C, the swarm diameter was 43.0 ± 3.4 mm for the wild-type strain. The motility of the *ybgF1* mutant was not significantly affected, that of the *ybgC1* *tolQ2*, *tolA1* and *tolA2* mutants was strongly reduced, while *tolB1* and *pal1* mutants did not move at all (Table 2). Addition of 10 % sucrose to the swarm agar plates partially restored the motility of the mutants (Table 2).

As the growth rate of the mutant strains could affect their motility, a more direct observation of fresh bacteria was carried out by phase-contrast microscopy, and cell motility was recorded with a camera. The results were in agreement with those observed on swarm plates. The wild-type strain moved rapidly, while the *tolQ* and *tolA* derivatives moved much more slowly, and the *tolB* and *pal* mutants did not move at all (data not shown).

Morphological characterization of the *Er. chrysanthemi* *tol-pal* mutants

The cells were examined by electron microscopy after negative staining of the cells (Fig. 3). After the growth of patches on LB plates, the wild-type strain was rod-shaped with a mean size of 2.1 × 0.65 µm and presented long flagella (Fig. 3A). Under the same conditions, the *tolQ2*

mutant presented an altered morphology with short twisted filaments (Fig. 3B). This could be due to the presence of square poles at one end of the cells, where the poles tended to grow to one side rather than at the middle of the bacteria (Fig. 3C). Bacteria had a few, short flagella. The *tolA2* mutant also formed short filaments and had a few, short flagella (Fig. 3D). The septum was not always at the middle of dividing bacteria, leading to cells of heterogeneous sizes (Fig. 3E). Vesicles could be observed, even at the septum (Fig. 3D). The *tolB1* mutant also formed short filaments, but had no flagella (Fig. 3F). The size of the cells and the position of the septa were highly irregular. The *pal1* mutant lacked flagella (Fig. 3G). Vesicles appeared at the cell surface as well as very large envelope excrescences.

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 and large envelope excrescences still appeared at the cell surface of the *pal1* mutant.

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*-chlorophenylhydrazone (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.*

Fig. 3. Transmission electron microscopy of *Er. chrysanthemi* 3937 and *tol-pal* mutants. Cells were grown overnight on LB plates and treated for electron microscopy as described in Methods. The vesicles are indicated by arrows.

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

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).

Strain	Maximal concentration that allowed growth		
	Sodium cholate (mg ml ⁻¹)	SDS (mg ml ⁻¹)	CCCP (µg ml ⁻¹)
3937	15	3	20
<i>ybgC1</i>	10	0.9	20
<i>tolQ2</i>	7.5	0.5	15
<i>tolA1</i>	7.5	0.5	15
<i>tolA2</i>	7.5	0.5	15
<i>tolB1</i>	7.5	0.2	10
<i>pal1</i>	7.5	0.2	10
<i>ybgF1</i>	10	3	20

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 & Glaeser, 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 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).

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.

Er. 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 pathogens. 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 phytopathogenic bacteria such as *Pseudomonas syringae* and *Er. chrysanthemi* (Loubens *et al.*, 1993; Mukhopadhyay *et al.*, 1988; Page *et al.*, 2001). Unlike the *tol-pal* mutants, the *opgGH* mutants show a complete loss of virulence, even on 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 the Rcs phosphorelay in the *opg* and *tol* mutants (Clavel *et al.*, 1996; Ebel *et al.*, 1997). Rcs is associated with virulence in an increasing number of bacteria, including *Erwinia amylovora* (Bereswill & Geider, 1997) and *S. enterica* (Cano *et al.*, 2002; Dominguez-Bernal *et al.*, 2004). In *E. coli*, the Rcs phosphorelay activates the genes necessary for capsule synthesis (Stout & Gottesman, 1990), and *ftsZ* and *ftsA* involved in cell division (Carballes *et al.*, 1999), and represses the genes involved in flagella synthesis (Francez-Charlot *et al.*, 2003). As in *E. coli*, some of the phenotypes observed in the *Er. chrysanthemi* *tol-pal* mutants, such as the defects in motility and cell division, could result from an activation of the Rcs phosphorelay. Therefore, the impaired virulence of the *tol-pal* mutants probably results from the combination of a defect in cell envelope integrity and an activation of regulatory networks, such as the Rcs phosphorelay.

E. coli *tol-pal* genes were unable to complement the corresponding *Er. chrysanthemi* mutations. Conversely, the *Er. chrysanthemi* *tol-pal* genes complemented the *E. coli* *tol-pal* mutations, despite the fact that the TolA protein was shorter in *Er. chrysanthemi* [as in *Erwinia carotovora* (data not shown)]. The possibility that *Er. chrysanthemi* *tol-pal* genes perform additional essential functions should be considered. This hypothesis can be assessed by the construction and analysis of selected hybrid Tol-Pal proteins, as most of the functional domains of the Tol-Pal proteins have been identified in *E. coli*. Another particularity of the *tol-pal* gene cluster in *Er. chrysanthemi* is the phenotype of the *ybgF* mutant in this bacterium. The *Er. chrysanthemi* *ybgF* mutant is sensitive to sodium cholate, a phenotype that has not been described in other species. Another intriguing property of this *ybgF* mutant is its apparent increase in pectate lyase activity. Further

examination of these phenotypes could help to clarify the role of 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 *Er. 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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