The enterobacterial pathogen *Erwinia chrysanthemi* 3937 produces a systemic soft rot in the African violet (*Saintpaulia ionantha*). The symptoms consist of a progressive disorganization of parenchymatous tissues following the release in plant walls of a set of bacterial pectinolytic enzymes including pectate lyases and pectin methylesterases (Barras et al., 1994; Lojkowska et al., 1995; Hugouvieux-Cotte-Pattat et al., 1996; Shevchik et al., 1997). Genetic and physiological studies have shown that the multiple isoforms of pectinases are regulated by several environmental signals, among which iron plays a major role *in planta* (Masclaux & Expert, 1995; Expert et al., 1996).

In response to iron starvation, *Er. chrysanthemi* 3937 synthesizes the catechol-type siderophore chrysobactin (Persmark et al., 1989). Iron acquisition mediated by chrysobactin is essential for the bacterium to disseminate throughout its host plant (Enard et al., 1988; Masclaux & Expert, 1995). Mutants affected in chrysobactin-mediated iron transport cannot use EDDHA [ethylenediamine-N,N′-bis(2-hydroxyphenylacetic acid)]-chelated iron as an iron source but retain the ability to grow on a medium containing 2,2′-dipyridyl (Enard et al., 1988). Growth on 2,2′-dipyridyl is dependent on a second iron-acquisition system based on the production of achromobactin, a siderophore which is neither a catechol nor a hydroxamate (Mühling et al., 2000). For instance, chrysobactin-deficient mutants lacking a functional ferriachromobactin permease encoded by the *cbrABCD* operon failed to grow in the presence of 2,2′-dipyridyl (Mühling et al., 1995). These two siderophore-dependent iron-transport pathways are differentially regulated by iron: derepression of the chrysobactin system requires more severe iron deficiency than that of the achromobactin system. Repression by iron is mediated by the ferric uptake regulatory protein,
Fur, that was recently characterized in Er. chrysanthemi 3937. The Fur repressor plays a key role in the coordinate regulation of genes encoding iron-transport proteins and pectinases (Franza et al., 1999). In addition, Er. chrysanthemi 3937 is able to use the two xenosidophores, enterobactin and ferrichrome, produced by enterobacteria and the fungal genus Ustilago.

Here, we describe a TonB mutant isolated by insertional mutagenesis of a chrysobactin-deficient strain. In Escherichia coli, the function of the TonB protein has been thoroughly studied (for reviews see Braun, 1995; Mocek & Coulton, 1998). The passage of the ferrisiderophore through the outer membrane requires active transport via a receptor which functions as a pore energized by the cytoplasmic-membrane-generated protonotive force transduced by the TonB protein and auxiliary proteins ExbB and ExbD forming the Ton complex (Higgs et al., 1998; Larsen et al., 1999; Postle, 1999). The Er. chrysanthemi TonB mutant studied displays a leaky phenotype regarding utilization of the ferric complex of achromobactin.

METHODS

Strains and media. Bacterial strains, phages and plasmids used in this work are described in Table 1. L-broth, L-agar medium and M63 medium (Miller, 1972) were used routinely. TA7 was used for soft agar overlays (Franza & Expert, 1991). Tris medium (Franza & Expert, 1991) was used for low-iron liquid cultures. Iron-depleted MM9 agar medium was used as described by Schwyn & Neilands (1987). Rich media were provided iron-rich conditions. When necessary, the following antibacterial agents were included in the media: kanamycin (Km), 25 µg ml⁻¹; streptomycin (Sm), 100 µg ml⁻¹; spectinomycin (Spc), 80 µg ml⁻¹; tetracycline (Tc), 10 µg ml⁻¹; ampicillin (Ap), 35 µg ml⁻¹. For E. coli cultures these concentrations were doubled. Amino acids and vitamins were added to a final concentration of 40 and 1 µg ml⁻¹, respectively. Carbon sources were added to a final concentration of 0.2 % (glucose and glycerol) and 0.1 % (polygalacturonate). Incubations were carried out at 30 °C for Er. chrysanthemi and 37 °C for E. coli. Glassware was decontaminated as described by Pugsley & Reeves (1976).

Isolation and phenotype analysis of mutants. Mutagenesis of Er. chrysanthemi strain 3937 cbsE1 was performed with the Mu dII1734 derivative as previously described (Enard et al., 1988). Kanamycin-resistant survivors were replicated onto CAS-agar medium (Schwyn & Neilands, 1987). CAS assays (Schwyn & Neilands, 1987) were also performed using culture supernatants of strains grown in Tris medium. Mutants displaying an altered phenotype on CAS-agar medium were streaked on L-agar medium supplemented with 2,2'-dipyridyl.

Cross-feeding assay. Utilization of ferriachromobactin, ferrichromobactin, ferrirrientobactin and ferrichrome by the tested strains was determined in a bioassay under low-iron conditions. Plates were poured with 25 ml EDDHA-L-agar medium seeded with an overnight L-broth culture of each strain at a final concentration of 10³ c.f.u. ml⁻¹. Ferriachromobactin, ferrichromobactin and ferrirrientobactin were provided as culture supernatants of strains 3937 cbsE1, 3937 cbsE1tonB60 and MM272-60, respectively. Sterile disks of 6 mm diameter were placed on the agar surface and 60 µl of each filter-sterilized fluid was added. The radii of zones of growth of the tested strains were measured after 48 h.

Mating, transduction and transformation methods. These were as described by Franzia et al. (1991).

DNA methods. The cosmid library of Er. chrysanthemi was constructed in vector pLA2917 linearized with BgIII using genomic DNA partially digested with Sau3A. The ligated DNA was packaged in vitro using the Gold Kit packaging extracts as recommended by the supplier (Stratagene). The packaged extracts were used to transduce E. coli ED8767. TcⅠ clones were selected and screened for kanamycin sensitivity. Other DNA methods were as previously described (Franza & Expert, 1991).

Transport experiments. Bacterial culture and transport experiments were performed as previously described (Mahé et al., 1995) with the following modifications: radiolabelled iron (1 µM) was supplied as ⁵⁵Fe-achromobactin (metal:ligand ratio of approximately 1:4). The source of achronobactin was a filter-sterilized supernatant of a culture of strain 3937 cbsE1tonB60 grown for 8 h in Tris medium. Formation of the ferric complex of achronobactin was estimated using the CAS assay. Filters were washed with 20 ml supernatant fluid containing a 10-fold excess of unlabelled Fe-achromobactin. Experiments were performed in duplicate.

Determination of pectate lyase activity in bacterial culture. Cultures were grown as previously described (Franza et al., 1999). Pectate lyase specific activity is expressed as µmol degradation products liberated min⁻¹ (mg dry weight of bacteria)⁻¹. Each experiment was performed three times.

Pathogenicity assay. Pathogenicity was tested on potted African violets (cv. Blue Rhapsody), as reported by Expert & Toussaint (1985) with modifications: the inoculum was 100 µl of a bacterial culture grown in M63 medium for 18 h diluted in the same medium to give an OD₆₆₀ of 0.3. Twelve plants were inoculated with each strain. Progression of the symptoms was scored for 5 weeks.

RESULTS AND DISCUSSION

Isolation and characterization of a TonB⁻ mutant

Strain 3937 cbsE1, which does not produce chrysobactin, but produces orange haloes on CAS-agar medium, was mutagenized by random insertion of the Mu dII1734 genome. Approximately 5000 Km² clones were screened for alteration of their iron-assimilation system by testing for changes in siderophore activity on CAS-agar medium. One mutant, 3937 tonB60, gave rise to orange haloes larger than those of the parental strain, suggesting an impaired transport system. This mutant was unable to grow on L-agar medium containing 2,2'-dipyridyl. To check the presence of a single insertion of the mini-Mu prophage, the mutation was transduced into the parental strain using the generalized transducing phage φEC2. A 75 % cotransduction frequency of the TonB⁻ phenotype...
and kanamycin resistance conferred by the reporter gene of the prophage was observed; this percentage corresponded to the cotransduction frequency for a single insertion. As Mu dIII1734 is able to generate lacZ translational fusions, we checked the Lac\(^{-}\) phenotype of the mutant by transducing the mutation into strain L2. The transductant had a Lac\(^{-}\) phenotype. The mutant grew normally on M63 agar medium. Utilization of ferrichromobactin by the mutant was tested in a cross-feeding assay, using the culture supernatant fluid of the

| Table 1. Bacterial strains, bacteriophages and plasmids |
|-----------------------------|-----------------------------|-----------------------------|
| **Strain, phage, plasmid**   | **Relevant characteristics**           | **Source/reference**         |
| Er. chrysanthemi 3937         | Wild-type, isolated from *Saintpaulia ionantha* | Kotoujansky *et al.* (1982) |
| 3937 cbsE1                   | *cbsE*:Ω, Cbs\(^{-}\) Spc\(^{-}\) Sm\(^{-}\) | Mahé *et al.* (1995)         |
| L2                          | Lac\(^{-}\) derivative of 3937 | Hugouvieux-Cotte-Pattat & Robert-Baudouy (1985) |
| A1076                       | *trp-1 bis-1 gal-1 thy-1, Sm\(^{-}\)* | S. Reverchon, LGMM, INSA, Villeurbanne, France |
| 3937 cbsE1 tonB60            | tonB60 mutation transduced into 3937 cbsE-1 | This work                     |
| 3937 tonB60                  | tonB60 mutation transduced into 3937 | This work                     |
| 3937 cbsE1 acs-37            | *acs-37* mutation transduced into 3937 cbsE1 | This work                     |
| 3937 cbsE1 acs-37 tonB60     | tonB60 mutation transduced into 3937 cbsE1 acs37 | This work                     |
| 3937 cbsE1 acs-48            | 3937 cbsE1 MuIII1734 lysogenic | This work                     |
| 3937 cbsE1 acs-59            | 3937 cbsE1 MuIII1734 lysogenic | This work                     |
| 3937 cbsE1 acs-64            | 3937 cbsE1 MuIII1734 lysogenic | This work                     |
| 3937 acs-59                  | *acs-59* mutation transduced into 3937 | This work                     |
| E. coli POI1734              | F\(^{-}\) araD139 ara\(_{3}\) (Mu cts3 Δ(lac)X74 galU galK rpsLMu dIl1734 supE44 supF58 hsdS3 (r\(_{E}\),m\(_{E}\)) recA56 galK2 galT22 metB1 | Castilho *et al.* (1984) |
| ED8767                       | supE44 supF58 hsdS3 (r\(_{E}\),m\(_{E}\)) recA56 galK2 | Murray *et al.* (1977)       |
| BR158                        | aroB thi malT tsx tonB | Hantke & Braun (1978)         |
| JM109                        | recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac–proAB) F\(^{-}\) [traD36 proAB\(^{+}\) lacI\(^{q}\) lacZAM15] | Yanisch-Perron *et al.* (1985) |
| MM272-60                     | F\(^{-}\) fes thi trpE proC leuB lacY mtl xyl | Pettis *et al.* (1988)       |
| C600                         | F\(^{-}\) thi thr-1 leuB lacY fmuA supE | Bachmann (1987)               |
| Bacteriophages               | Mu dIII1734 Mu cts62 Δ(Mu AB) Km\(^{-}\) Δ(lac‘ZYA) | Castilho *et al.* (1984) |
| Mu dIlPR13                   | Mu cts62 Δ(Mu AB) Km\(^{-}\) Δ(lac‘ZYA) | Faelen (1987)                 |
| Mu cts62                     | Thermoinducible | Howe (1973)                   |
| μEC2                         | Generalized transducing phage from *Er. chrysanthemi* strain 3690 | Résoibois *et al.* (1984)    |
| Plasmids/cosmids             | pUC18 High copy number, Ap\(^{+}\) | Yanisch-Perron *et al.* (1985) |
|                             | pUC18 carrying the 12 kb EcoRI fragment, Ap\(^{+}\) | Expert *et al.* (1992)       |
|                             | pRLK2013 Mobilization helper plasmid Km\(^{-}\) | Figurski & Helinski (1979)    |
|                             | pLA2917 21 kb mobilizable cosmid, Te\(^{+}\) Km\(^{-}\) | Allen & Hanson (1985)        |
|                             | pLA2917 tonB from *Er. chrysanthemi* strain 3937, Te\(^{+}\) Km\(^{-}\) | This work                     |
|                             | pULB110 RP4 Km::Mu3A, Te\(^{+}\) Ap\(^{+}\) Km\(^{-}\) | Van Gijsegem & Toussaint (1982) |
|                             | pCE1 pUC18 carrying the 8 kb *BamHI* tonB fragment from pLA2C2, Ap\(^{+}\) | This work                     |
|                             | pCE2 pUC18 carrying the 6–2 kb *BamHI* fragment Km\(^{-}\) from 3937 cbsE1 fst-60, Ap\(^{+}\) | This work                     |
Terminal part of the Mu

To identify the gene affected in the mutant tonB60, we first isolated the host DNA sequence flanking the left terminal part of the Mu dIII1734 prophage responsible for the mutation. Total DNA digest from the mutant strain was digested with BamHI and ligated into pUC18 DNA linearized with the same enzyme. After electroporation of E. coli JM109 cells, one Km\(^r\) Ap\(^r\) transformant was recovered and its plasmid content analysed. The recombinant plasmid pCE2, harbouring an insert of 6.2 kb including both host and phage DNA, was identified. This DNA fragment was then used as a DNA–DNA hybridization probe to screen a wild-type gene library constructed in the low-copy-number cosmid pLA2917. Five cosmids hybridized with the probe. Each cosmids was mobilized into the strain harbouring the tonB60 mutation. Three of the five cosmids were able to restore the wild-type phenotype to the mutant (growth on 2,2′-dipyridyl L-agar plates, normal size of orange halo on CAS-agar medium and utilization of the four siderophores as iron sources). The 6.2 kb DNA fragment was then used as a DNA–DNA hybridization probe to identify the BamHI restriction fragment in the wild-type genomic DNA. It was an 8.5 kb fragment also present in the three cosmids which complemented the mutation. The three cosmids carry genomic inserts of 20–25 kb. The smallest, pL4C2, was further studied. Cosmid pL4C2 was mobilized in the E. coli TonB\(^–\) mutant BR158. A cross-feeding assay using enterobactin showed that the transconjugant was functionally complemented (data not shown). The 8.5 kb BamHI fragment was inserted into pUC18, generating pCE1. pCE1, like cosmid pL4C2, was able to restore the wild-type phenotype to the mutant 3937 tonB60 and the E. coli TonB\(^–\) mutant.

**DNA sequence of the Er. chrysanthemi tonB gene and organization of the tonB region**

Plasmid pCE1 was used for sequencing (GenBank accession no. Y15888). The 1122 bp sequenced in the genomic insert included an ORF of 759 bp (nucleotide positions 302–1060) encoding a putative protein of 252 amino acids with a predicted molecular mass of 27600 Da. The deduced translation product is 58% identical and 72% similar to the *Serratia marcescens* TonB protein. The amino acid sequence is rich in proline (19%), with nearly half the residues from amino acid positions 72 to 118 being proline. The region includes a (Lys-Pro)\(_3\)-(Glu-Pro)-(Lys-Pro)\(_2\) repeat. This Pro-rich region is typical of TonB proteins and confers a rod-like structure to the protein such that it can reach the outer membrane from its anchor in the inner membrane (Hannavy *et al*, 1990; Larsen *et al*, 1993). This anchor is located at the N-terminal end of the predicted protein (amino acids 15–35, Karlsson *et al*, 1993a, b; Postle & Skare, 1988) and harbours the highly conserved motif SHLS (Koebnik, 1993; Koebnik *et al*, 1993) known to be essential in *E. coli* for interaction with the auxiliary protein ExbB of the Ton complex (Braun *et al*, 1996; Traub *et al*, 1993). The central part of the predicted protein contains the highly conserved motif PXYP, which is thought to be required for the protein to adopt the conformation necessary to allow interaction with the outer membrane receptors of the ferrisidero-
TonB in Erwinia chrysanthemi 3937

![Genetic organization of the Er. chrysanthemi tonB region.](image)

**Genetic mapping of the tonB60 mutation**

To locate the tonB gene on the linkage map of *Er. chrysanthemi* 3937, we used the kanamycin-sensitive conjugative plasmid pULB110, derived from RP4:miniMu. The cotransfer of the *trp* marker and the bacteriophage φ80 attachment site (70% DNA sequence identity on one strand; Fig. 2b). Nevertheless, strain 3937 is not sensitive to φ80.

Twenty-four bases downstream from the stop codon, there is a 14 nucleotide sequence repeated in the opposite orientation, thus forming an inverted repeat typical of a rho-independent transcription terminator. This potential terminator may work in both directions in *Er. chrysanthemi*, as has been demonstrated in *E. coli* (Postle & Good, 1985). No ORF appears to start immediately downstream from the putative tonB gene ORF, but the end of a truncated ORF was identified on one strand. The 136 base stretch of this truncated ORF shares 66% identity with the *E. coli* p14 gene and the encoded amino acid sequences are 70% identical (Fig. 2b; Postle & Good, 1985). A p14 homologue has also been identified next to the tonB gene in three other enterobacteria (Gaisser & Braun, 1991; Hannavy et al., 1990; Koebnik et al., 1993).

The location of the prophage insertion in the tonB gene was determined using pCE2. The insertion is 48 bp downstream from the translation start. The position of this insertion excluded the possibility of translation of a functional truncated TonB protein that could explain the residual growth of the mutant observed in cross-feeding assays.
kanamycin marker of the mutation was 96\%, suggesting possible cotransduction of the two markers with the phage \(\phi\)ECII. An average of 45\% cotransduction was obtained. Therefore, the \(tonB\) gene maps very close to the \(trp\) marker, less than 62 kb away (the size of the phage genome). For a map of strain 3937 see Hugouvieux-Cotte-Pattat \textit{et al.} (1996).

**Pathogenicity of the \(TonB^−\) mutant**

The pathogenicity of the \(TonB^−\) mutant was assessed using potted African violets. Unlike the parental strain, which gave rise to a systemic infection on a quarter of the inoculated plants, the mutant strain had no systemic effects. However, the mutant was able to macerate the inoculated leaf (two-thirds of the inoculated plants), as has been observed for mutants affected in chrysobactin inoculated leaf (two-thirds of the inoculated plants), as has been observed for mutants affected in chrysobactin biosynthesis (Enard \textit{et al.}, 1988). It is surprising that a strain deficient in all high-affinity iron-uptake pathways was not less aggressive than a mutant affected in the chrysobactin system only. No revertants were recovered from inoculated leaves (data not shown) and thus the ability of the \(TonB^−\) mutant to invade the leaf did not result from the reversion of the \(tonB\) mutation. We determined the total pectate lyase activity of the \(tonB\) mutant, and for comparison, the wild-type strain. Both strains were grown under various conditions of iron availability, i.e. in Tris medium with or without EDDHA, a strong ferric iron chelator. The carbon source was either glycerol or polygalacturonate, the latter being the inducer of genes encoding pectinases. The \(tonB\) mutant grew slightly slower than the wild-type in Tris medium (Fig. 3a). Therefore, although poor in iron because of the low phosphate concentration, Tris medium contains traces of iron allowing the \(tonB\) mutant to grow. However, in the presence of EDDHA, the growth of the mutant strain was almost abolished (data not shown). In the presence of glycerol no pectate lyase activity was detected. In the presence of the pectic inducer polygalacturonate, the pectate lyase activity produced by the \(tonB\) mutant was twice as high as that of the wild-type strain and the activity of the mutant appeared earlier (Fig. 3b). Increase in this enzymic activity may explain why the virulence of the mutant was not as severely impaired as expected.

**Conclusions**

This study was intended to provide further elucidation of iron transport in \textit{Er. chrysanthemi}. Insertional mutagenesis using a chrysobactin-deficient strain allowed us to isolate a \(TonB^−\) mutant. The predicted product of the \textit{Er. chrysanthemi} \(tonB\) gene displays the typical features of TonB proteins. Essential for the supply of energy to ferrisiderophore receptors, TonB proteins are found in many Gram-negative bacteria. Genetic complementation showed that the TonB protein from \textit{Er. chrysanthemi} can functionally replace the TonB protein of \textit{E. coli}. The genetic organization of the \textit{Er. chrysanthemi} \(tonB\) gene was similar to that in \textit{E. coli}. Moreover, the \(tonB\) gene maps close to the \(trp\) marker, as in both \textit{E. coli} and \textit{S. typhimurium}. As in the genomes of other enterobacteria, no \textit{exbB} and \textit{exbD} genes were found next to the \(tonB\) gene of \textit{Er. chrysanthemi}.

The apparent discrepancy in the behaviour of the \(TonB^−\) mutant in the cross-feeding assays and the uptake experiments is interesting. In a cross-feeding assay, bacterial growth is recorded after 24 h incubation. Possibly, the relatively long period of time allows expression of a \(TonB\)-independent transport mechanism that cannot be detected during the uptake experiment. Similarly, \(TonB\)-independent transport of ferrioxamines B and E has been described in \textit{Salmonella enterica}. This transport was only revealed by cross-feeding assays and its level has not been determined (Kingsley \textit{et al.}, 1999).
Finally, the TonB\(^{-}\) mutant was not strongly impaired in its ability to invade leaf tissues following inoculation. The total pectate lyase activity produced by bacterial cells grown under iron-deficient conditions was twice as high in the mutant strain as in the wild-type. Previous work (Franza et al., 1999) demonstrating the existence of coordinate regulation, by iron via the Fur sensory and regulatory protein, between iron transport functions and synthesis of several pectate lyases may explain this effect. Depletion of the intracellular iron pool, as probably occurs in the TonB\(^{-}\) mutant, could result in up-regulation of iron-controlled genes encoding pectate lyase.

ACKNOWLEDGEMENTS

We thank Martine Boccara for the gift of the cosmid library, Muriel Feau for her help in the genetic mapping and Alex Edelman for reading of the English of the manuscript. This work was supported by grants from the Institut National de la Recherche Agronomique (INRA). D.E. is a researcher from the Centre National de la Recherche Scientifique (CNRS).

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Received 25 October 1999; revised 28 February 2000; accepted 15 May 2000.