Characterization of a *tonB* mutation in *Erwinia chrysanthemi* 3937: TonB_{Ech} is a member of the enterobacterial TonB family

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The pectinolytic enterobacterium *Erwinia chrysanthemi* 3937 causes a systemic disease in its natural host, the African violet (*Saintpaulia ionantha*). It produces two structurally unrelated siderophores, chrysobactin and achromobactin. Chrysobactin makes a large contribution to invasive growth of the bacterium in its host. Insertion mutants of a chrysobactin-defective strain were constructed and screened on the universal CAS-agar medium used for siderophore detection. A set of mutants affected in the production of achromobactin were identified. This paper describes a mutant affected in the transport of all the ferrisiderophores used by the bacterium as iron sources. Molecular analysis revealed that the insertion mutation disrupts the *tonB* gene. The predicted *Er. chrysanthemi* TonB protein has a molecular mass of 27 600 Da and shares 20–58% identity with the TonB proteins from 20 other bacterial species. The pathogenicity of the *tonB* mutant was assessed by inoculation of African violets. The impairment in the spread of symptoms was similar in the *tonB* mutant to that in chrysobactin-defective mutants. However, the pectinolytic activity, the major pathogenicity determinant in *Er. chrysanthemi*, appeared to be stimulated twofold in the *tonB* mutant.

Keywords: iron transport, siderophore, achromobactin, chrysobactin, pathogenicity

INTRODUCTION

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-hydroxyphenyl-acetic 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 (Mahé *et al.*, 1995) but a citrate derivative (Münzinger *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 (Mahé *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,

Abbreviations: EDDHA, ethylenediamine-*N,N'*-bis(2-hydroxyphenyl-acetic acid).

The GenBank accession number for the sequence reported in this paper is Y15888.

Fur, that was recently characterized in *Er. chrysanthemi* 3937. The Fur repressor plays a key role in the co-ordinate regulation of genes encoding iron-transport proteins and pectinases (Franza *et al.*, 1999). In addition, *Er. chrysanthemi* 3937 is able to use the two xenosiderophores, 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; Moeck & 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 protonmotive 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 made iron-poor by addition of chelators: EDDHA (Sigma) deferrated by the procedure of Rogers (1973) to a final concentration of 270 and 540 µM for cultures of *Er. chrysanthemi* and *E. coli*, respectively; 2,2'-dipyridyl (Sigma) to a final concentration of 200 µM. In Tris medium, EDDHA was added to a final concentration of 13 µM. Addition of ferric chloride to Tris medium to a final concentration of 20 µM provided iron-rich conditions. When necessary, the following antibacterial agents were included in the media: kanamycin (Km), 25 µg ml⁻¹; chloramphenicol (Cm), 10 µg ml⁻¹; streptomycin (Sm), 100 µg ml⁻¹; spectinomycin (Sp), 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 deferrated 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, ferri-chrysobactin, ferrienterobactin and ferrichrome by the tested strains was determined in a bioassay under low-iron con-

ditions. 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 10⁴ c.f.u. ml⁻¹. Ferriachromobactin, ferri-chrysobactin and ferrienterobactin were provided as culture supernatants of strains 3937 *cbsE1*, 3937 *acs-59* 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 Franza *et al.* (1991).

DNA methods. The cosmid library of *Er. chrysanthemi* was constructed in vector pLA2917 linearized with *Bgl*II using genomic DNA partially digested with *Sau*3A. 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. Tc^r 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 ⁵⁹Fe-achromobactin (metal:ligand ratio of approximately 1:4). The source of achromobactin 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 achromobactin 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 OD₆₀₀ 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 Km^r 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

Table 1. Bacterial strains, bacteriophages and plasmids

Strain, phage, plasmid	Relevant characteristics	Source/reference
<i>Er. chrysanthemi</i>		
3937	Wild-type, isolated from <i>Saintpaulia ionantha</i>	Kotoujansky <i>et al.</i> (1982)
3937 <i>cbsE1</i>	<i>cbsE::</i> Ω, Cbs ⁻ Spc ^r Sm ^r	Mahé <i>et al.</i> (1995)
L2	Lac ⁻ derivative of 3937	Hugouvieux-Cotte-Pattat & Robert-Baudouy (1985)
A1076	<i>trp-1 his-1 gal-1 thy-1</i> , Sm ^r	S. Reverchon, LGMM, INSA, Villeurbanne, France
3937 <i>cbsE1 tonB60</i>	<i>tonB60</i> mutation transduced into 3937 <i>cbsE-1</i>	This work
3937 <i>tonB60</i>	<i>tonB60</i> mutation transduced into 3937	This work
3937 <i>cbsE1 acs-37</i>	<i>acs-37</i> mutation transduced into 3937 <i>cbsE1</i>	This work
3937 <i>cbsE1 acs-37 tonB60</i>	<i>tonB60</i> mutation transduced into 3937 <i>cbsE1 acs37</i>	This work
3937 <i>cbsE1 acr-48</i>	3937 <i>cbsE1</i> MudIII1734 lysogenic	This work
3937 <i>cbsE1 acs-59</i>	3937 <i>cbsE1</i> MudIII1734 lysogenic	This work
3937 <i>cbsE1 acs-64</i>	3937 <i>cbsE1</i> MudIII1734 lysogenic	This work
3937 <i>acs-59</i>	<i>acs-59</i> mutation transduced into 3937	This work
<i>E. coli</i>		
POII1734	F ⁻ <i>araD139 ara::</i> (Mu cts)3 Δ(<i>lac</i>)X74 <i>galU galK rpsLMu dII1734</i>	Castilho <i>et al.</i> (1984)
ED8767	<i>supE44 supF58 hsdS3</i> (r _B ⁻ m _B ⁻) <i>recA56 galK2 galT22 metB1</i>	Murray <i>et al.</i> (1977)
BR158	<i>aroB thi malT tsx tonB</i>	Hantke & Braun (1978)
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi</i> Δ(<i>lac-proAB</i>) F' [<i>traD36 proAB⁺ lacI^q lacZΔM15</i>]	Yanisch-Perron <i>et al.</i> (1985)
MM272-60	F ⁻ <i>fes thi trpE proC leuB lacY mtl xyl</i>	Pettis <i>et al.</i> (1988)
C600	F ⁻ <i>thi thr-1 leuB lacY fhuA supE</i>	Bachmann (1987)
Bacteriophages		
Mu dII1734	Mu cts62 Δ(Mu AB) Km ^r Δ(<i>lac'</i> ZYA)	Castilho <i>et al.</i> (1984)
Mu dIIpR13	Mu cts62 Δ(Mu AB) Cm ^r Δ(<i>lac'</i> ZYA)	Faelen (1987)
Mu cts62	Thermoinducible	Howe (1973)
φEC2	Generalized transducing phage from <i>Er. chrysanthemi</i> strain 3690	Résibois <i>et al.</i> (1984)
Plasmids/cosmids		
pUC18	High copy number, Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
pDE4	pUC18 carrying the 12 kb <i>EcoRI</i> fragment, Ap ^r	Expert <i>et al.</i> (1992)
pRK2013	Mobilization helper plasmid Km ^r	Figurski & Helinski (1979)
pLA2917	21 kb mobilizable cosmid, Tc ^r Km ^r	Allen & Hanson (1985)
pL4C2	pLA2917 <i>tonB</i> from <i>Er. chrysanthemi</i> 3937, Tc ^r Km ^s	This work
pULB110	RP4 Km ^r ::Mu3A, Tc ^r Ap ^r Km ^s	Van Gijsegem & Toussaint (1982)
pCE1	pUC18 carrying the 8 kb <i>Bam</i> HI <i>tonB</i> fragment from pL4C2, Ap ^r	This work
pCE2	pUC18 carrying the 6.2 kb <i>Bam</i> HI fragment Km ^r from 3937 <i>cbsE1 fst-60</i> , Ap ^r	This work

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 dII1734 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 ferri-chromobactin by the mutant was tested in a cross-feeding assay, using the culture supernatant fluid of the

Fig. 1. Transport of ^{59}Fe -achromobactin into mutant strains. Bacterial cells were grown in Tris medium prior to the assay. Symbols indicate the different genetic backgrounds of 3937 cells used: ●, (3937 *cbsE1 acs-37*); ○, (3937 *cbsE1 acs-37 tonB60*); □, (3937 *cbsE1 acr-48*). Results are from one representative experiment.

parental strain grown in Tris medium. For this purpose, the mutation was transduced into the achromobactin-deficient strain 3937 *cbsE1 acs-37*. The presence of the mutation resulted in the reduction of the size of the halo of growth (radius of 4–5 mm instead of 6–7 mm) but unlike mutation *acr-48*, disrupting the synthesis of the ferriachromobactin receptor (Mahé *et al.*, 1995), did not completely abolish growth. Moreover, using the culture supernatants of strains 3937 *cbsE1 acs-37*, 3937 *cbsE1 acs-59* and 3937 *cbsE1 acs-64* grown in Tris medium, which do not produce achromobactin, haloes of growth of the mutant were never observed.

To investigate the level of ferriachromobactin transport in more detail, we carried out uptake experiments using ^{59}Fe -achromobactin (Fig. 1). The strain harbouring the *tonB60* mutation, like the receptor mutant, failed to incorporate radioactive iron. The parental strain incorporated 40 pmol ferric iron (a quarter of the labelled iron present in the assay) within 20 min. Thus, the mutation affects the transport of ferriachromobactin. We also checked the ability of the mutant to utilize chrysobactin, enterobactin and ferrichrome as iron sources. No halo of growth was observed in cross-feeding assays for any of these three siderophores. Therefore, the transport step affected by the *tonB60* mutation is required for the utilization of all siderophores taken up by the bacterium.

Cloning a parental genomic region complementing the *tonB60* mutation

To identify the gene affected in the mutant *tonB60*, we first isolated the host DNA sequence flanking the left terminal part of the Mu *dII1734* prophage responsible

for the mutation. Total DNA digest from the mutant strain was digested with *Bam*HI 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 cosmid 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 *Bam*HI 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 *Bam*HI 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 27 600 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-

Fig. 3. Growth and pectate lyase activity of strain 3937 (circles) and its TonB⁻ derivative (squares) grown under conditions of iron deficiency. (a) Growth curves in Tris medium with glycerol (left) or polygalacturonate (PGA; right) as carbon source, supplemented (open circles) or not supplemented (filled circles) with EDDHA. (Growth of the TonB mutant was almost abolished in EDDHA-supplemented medium, and is not shown.) (b) Pectate lyase specific activity [μmol degradation products liberated min^{-1} (mg dry weight of bacteria) $^{-1}$] determined in supernatants from cultures grown in Tris medium. Experiments were carried out in triplicate and standard deviations are shown.

kanamycin marker of the mutation was 96 %, suggesting possible cotransduction of the two markers with the phage ϕECII . 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 *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 biosynthesis (Enard *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 *Er. chrysanthemi*. Insertional mutagenesis using a chrysobactin-deficient strain allowed us to isolate a TonB⁻ mutant. The predicted product of the *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 *Er. chrysanthemi* can functionally replace the TonB protein of *E. coli*. The genetic organization of the *Er. chrysanthemi tonB* gene was similar to that in *E. coli*. Moreover the *tonB_{Ech}* gene maps close to the *trp* marker, as in both *E. coli* and *S. typhimurium*. As in the genomes of other enterobacteria, no *exbB* and *exbD* genes were found next to the *tonB* gene of *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 *Salmonella enterica*. This transport was only revealed by cross-feeding assays and its level has not been determined (Kingsley *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.

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