The *Pseudomonas aeruginosa* *tonB* gene encodes a novel TonB protein

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The *Pseudomonas aeruginosa* *tonB* gene was cloned by complementation of the *tonB* mutation of *Pseudomonas putida* strain TE516 (W. Bitter, J. Tommassen & P. J. Weisbeek, 1993, *Mol Microbiol* 7, 117–130). The gene was 1025 bp in length, capable of encoding a protein of 36860 Da. As with previously described TonB proteins, the *P. aeruginosa* TonB (TonB_{*pa*}) was rich in Pro residues (18.1%) and contained Glu-Pro/Lys-Pro repeats. Unlike previously described TonB proteins, however, TonB_{*pa*} lacked an N-terminal membrane anchor (signal) sequence and contained, instead, a predicted internal signal/anchor sequence, expected to yield an atypical N-terminal cytoplasmic domain in this protein. TonB proteins are essential components in iron–siderophore uptake in bacteria, apparently functioning as energy transducers in coupling the energized state of the cytoplasmic membrane to outer-membrane receptor function. As expected, *tonB* derivatives of *P. aeruginosa* were defective in siderophore-mediated iron acquisition. *tonB* gene expression was inducible by iron-limitation, consistent with the identification of a Fur consensus binding sequence upstream of the gene. TonB_{*pa*} showed substantially greater similarity to the *Escherichia coli* TonB protein than the *Pseudomonas putida* protein (31% identity vs. 20% identity) and *tonB_{pa}* was able to complement deficiencies in the acquisition of ferric enterobactin and vitamin B₁₂, and sensitivity to phage ϕ 80 of an *E. coli* *tonB* strain. The larger size of TonB_{*pa*} and its ability to function in both *E. coli* and *P. putida* make it a unique TonB protein whose characterization should enhance our understanding of TonB function in bacteria.

Keywords: *Pseudomonas aeruginosa*, iron transport, siderophore, *tonB*

INTRODUCTION

Energy-dependent receptor-mediated ligand uptake across the outer membrane of Gram-negative bacteria is dependent upon the function of the product of the *tonB* gene (Postle, 1990a, 1993). This Pro-rich protein is, in *Escherichia coli*, anchored to the cytoplasmic membrane via its N terminus and extends into the periplasm (Postle & Skare, 1988; Roof *et al.*, 1991; Hannavy *et al.*, 1990) where it appears to interact with receptor proteins in the outer membrane (Heller *et al.*, 1988; Günter & Braun, 1990;

Bell *et al.*, 1990; Skare *et al.*, 1993; Schöffler & Braun, 1989). This disposition of the protein reflects its apparent role in coupling the energized state of the cytoplasmic membrane to the outer-membrane receptors (Postle, 1990a, 1993). Current models of TonB-dependent receptors describe them as gated porins whose activities are modulated by the action of the TonB protein (Rutz *et al.*, 1992; Killmann *et al.*, 1993). These receptors, which include receptors for ferric siderophores (Braun *et al.*, 1987), vitamin B₁₂ (Heller & Kadner, 1985), haem/haemin (Jarosik *et al.*, 1994; Stojiljkovic & Hantke, 1992; Henderson & Payne, 1994) and transferrin (Jarosik *et al.*, 1995; Cornelisson *et al.*, 1992), show conserved regions of homology (Lundrigan & Kadner, 1986), one of which, the so-called TonB box (Schramm *et al.*, 1987), is proposed to interact directly with TonB (Heller *et al.*, 1988; Bell *et al.*, 1990). Such interaction appears to be mediated by a region of the TonB protein at or near residue Glu-160

Abbreviations: BHI, Brain Heart Infusion; EDDHA, ethylenediamine di(o-hydroxyphenylacetic acid); TonB_{*ec*}, TonB of *Escherichia coli*; *tonB_{pa}*/TonB_{*pa*}, *tonB*/TonB of *Pseudomonas aeruginosa*.

The GenBank accession number for the *tonB_{pa}* nucleotide sequence reported in this paper is U23764.

(Heller *et al.*, 1988; Bell *et al.*, 1990) or by the C terminus (Larsen *et al.*, 1993) which is required for TonB activity (Anton & Heller, 1991; Bruske & Heller, 1993). The Pro-rich domain of TonB, which is responsible for the anomalous migration of the protein on SDS-polyacrylamide gels (Larsen *et al.*, 1993), is predicted to adopt an elongated, structurally constrained conformation (Evans *et al.*, 1986; Brewer *et al.*, 1990) consistent with an apparent role in extending downstream/C-terminal TonB sequences across the periplasmic space where they can interact with receptor molecules (Larsen *et al.*, 1993).

Full TonB function in *E. coli* is dependent upon the products of the *exbB* and *exbD* genes (Guterman & Dann, 1973; Hantke & Zimmermann, 1981; Eick-Helmerich & Braun, 1989) found in the cytoplasmic membrane (Kampfenkel & Braun, 1992; Kampfenkel & Braun, 1993). The former is involved both in the stabilization of TonB (Karlsson *et al.*, 1993b; Skare & Postle, 1991; Fischer *et al.*, 1989), apparently via an interaction with the TonB N terminus (Larsen *et al.*, 1994; Karlsson *et al.*, 1993a; Jaskula *et al.*, 1994; Traub *et al.*, 1993), and in the energy transduction process itself (Skare *et al.*, 1993). Both *tonB* (Postle & Good, 1983; Bruske & Heller, 1993) and the *exb* (Eick-Helmerich & Braun, 1989) genes are preceded by Fur consensus binding sequences, consistent with the observed increase in expression of these genes under conditions of iron-limitation (Postle, 1990b; Hantke & Zimmermann, 1981).

tonB genes have been identified and characterized in a number of bacteria including *Salmonella typhimurium* (Hannavy *et al.*, 1990), *Serratia marcescens* (Gaisser & Braun, 1991), *Enterobacter aerogenes* (Bruske & Heller, 1993), *Klebsiella pneumoniae* (Bruske *et al.*, 1993), *Yersinia enterocolitica* (Koebnik *et al.*, 1993), *Pseudomonas putida* (Bitter *et al.*, 1993), *Haemophilus influenzae* (Jarosik *et al.*, 1994) and *Haemophilus ducreyi* (Klesney-Tait *et al.*, 1994). With the exception of the proteins from *Haemophilus* spp. (30–31% identity) and *P. putida* (28% identity), the TonB proteins exhibit a very high degree of sequence similarity (56–95% identity) with the *E. coli* TonB protein (TonB_{E.c.}). While the *tonB* gene is not linked to *exbB* and *exbD* homologues in most of the bacteria where they have been identified, the three genes appear to form an operon in *P. putida* and *H. influenzae* (Bitter *et al.*, 1993; Jarosik *et al.*, 1994).

P. aeruginosa expresses a number of outer-membrane proteins with homology to TonB-dependent receptors, including receptors for the ferric complexes of the siderophores pyoverdine (Poole *et al.*, 1993c), pyochelin (Heinrichs *et al.*, 1991; Ankenbauer & Quan, 1994) and enterobactin (Dean & Poole, 1993a). This homology and the fact that the *P. aeruginosa* ferric enterobactin receptor PfeA is active in *E. coli* (Dean & Poole, 1993a) suggests that receptor-mediated ferric siderophore uptake in *P. aeruginosa* utilizes a TonB-like system and, therefore, that a *tonB* homologue exists in this organism. In this report we identify and characterize a *P. aeruginosa tonB* gene (*tonB_{P.a.}*) which can functionally replace the *tonB* genes of *E. coli* and *P. putida*.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are described in Table 1. Spontaneous streptomycin-resistant derivatives of *P. aeruginosa* were isolated by harvesting 5 ml of an overnight L-broth culture and plating the pellet (resuspended in 100 µl) on L-agar containing 1000 µg streptomycin ml⁻¹. Distinct colonies appearing after 24–48 h incubation at 37 °C were recovered. For the construction of pTON-6, a ca 1.4 kb *Bam*HI fragment of pTON-4 carrying the 5' end of *tonB* and upstream sequences was cloned into the unique *Bgl*II site of pMP190 such that *tonB* was in the same orientation as the downstream, promoterless *lacZ* gene of this vector (Fig. 1). In constructing pTON-11, a ca 1.2 kb *Mse*I–*Bsr*BI fragment of pTON-4, also carrying the 5' end of *tonB* and upstream sequences, was cloned into the unique *Sma*I site of pPZ30, such that *tonB* was in the same orientation and in-frame with the downstream *lacZ* gene of this vector (Fig. 1).

Growth conditions. The iron-deficient minimal medium used has been described previously (Poole *et al.*, 1991). Succinate was replaced with glucose (0.4%, w/v) in this medium when culturing *E. coli* cells. Arginine-HCl (1 mM) and methionine (1 mM) were included in minimal medium as needed. L-broth (Poole *et al.*, 1990) or, where indicated, Brain Heart Infusion (BHI, Difco) were employed as the rich media. Antibiotics, including ampicillin (100 µg ml⁻¹), streptomycin (500 µg ml⁻¹, or as indicated) chloramphenicol (30 µg ml⁻¹, *E. coli*; 200 µg ml⁻¹, *P. aeruginosa*), tetracycline [10 µg ml⁻¹, *E. coli*; 25 µg ml⁻¹ (minimal medium) or 40 µg ml⁻¹ (rich medium), *P. putida*], nalidixic acid (25 µg ml⁻¹) and HgCl₂ (15 µg ml⁻¹) were included in growth media as appropriate. Solid media were obtained by the addition of Agar-agar (BDH) at 1.5% (w/v). Bacteria were cultured at 37 °C (*E. coli* and *P. aeruginosa*) or 30 °C (*P. putida*).

DNA methods. Plasmid DNA was routinely prepared by the alkaline lysis procedure (Sambrook *et al.*, 1989). For sequencing purposes the DNA was purified on CsCl gradients (Sambrook *et al.*, 1989) or using the Wizard Minipreps DNA Purification System (Promega) as outlined by the manufacturer. Restriction endonucleases and T4 ligase were obtained from Life Technologies or NEB and used according to the manufacturers' instructions or as described by Sambrook *et al.* (1989). Transformation of *E. coli* (Sambrook *et al.*, 1989) and *P. aeruginosa* (Berry & Kropinski, 1986) with plasmid DNA were performed as described. Restriction fragments were isolated, as required, from agarose gels (0.8–1.5%, w/v) using the Prep-a-gene system (Bio-Rad) as directed by the manufacturer. Introduction of plasmid (pRK415 and its derivatives) (Fig. 1) or cosmid (pLAFR3 library) (see below) DNA into *P. putida* required a triparental mating procedure employing the helper vector pRK2013 (Figurski & Helinski, 1979). Briefly, 100 µl of overnight cultures of plasmid-/cosmid-containing *E. coli* 5K or HB101, pRK2013-containing *E. coli* MM294 and *P. putida* TE156 were pelleted together in a microfuge tube, resuspended in 25 µl L-broth and spotted onto the centre of an L-agar plate. Following incubation overnight at 30 °C, bacterial growth was resuspended in 1 ml L-broth, plated on L-agar containing tetracycline (to select for plasmid-/cosmid-containing TE156) and nalidixic acid (to select against the *E. coli* strains) and incubated for 1–2 d at 30 °C.

Construction of a pLAFR3 library of *P. aeruginosa* PAO1 chromosomal DNA. Chromosomal DNA of *P. aeruginosa* PAO1, prepared by the method of Marmur (1961), was subjected to partial *Sau*3A digestion and fractionated on a sucrose gradient as described (Sambrook *et al.*, 1989). Fractions enriched for DNA of 20 kb in length (as determined by agarose gel electrophoresis)

Table 1. Bacterial strains and plasmids

Strain/plasmid	Description/phenotype*	Source/reference
<i>E. coli</i>		
5K	<i>thr lacZ rpsL thi ser hsdR hsdM</i>	Poole <i>et al.</i> (1988)
MM294	<i>supE44 λ⁻ rfbD1 spoT thi-1 endA1 hsdR17 pro</i>	Neidhardt (1987)
HB101	F ⁻ <i>hsdS20 (r_B⁻ m_B⁻) recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44 λ⁻</i>	Neidhardt (1987)
RK5173	F ⁻ Δ (<i>argF-lac</i>)U169 <i>araD139 relA1 rpsL150 ffb-5301 deoC1 thi gyrA219 non metE70</i>	Heller <i>et al.</i> (1988)
RK5015	RK5173 Δ <i>tonB</i>	Heller <i>et al.</i> (1988)
<i>P. putida</i>		
WCS358	Wild-type rhizosphere strain	Bitter <i>et al.</i> (1993)
TE156	WCS358 <i>tonB::Tn5</i>	Bitter <i>et al.</i> (1993)
<i>P. aeruginosa</i>		
K199	PAO1 wild-type	
K335	Spontaneous streptomycin-resistant derivative of K199	This study
PAO6609	<i>met9011 amiE200 rpsL pvd9</i>	Hohnadel <i>et al.</i> (1986)
K372	Derivative of PAO6609 deficient in pyochelin and the ferric pyochelin receptor FptA; probable <i>pchR</i> mutant	Heinrichs <i>et al.</i> (1991); Heinrichs & Poole (1993)
K883	PAO6609 <i>tonB::ΩHg</i>	This study
K886	K372 <i>tonB::ΩHg</i>	This study
Plasmids		
pLAFR3	Broad-host-range cosmid cloning vector; Tc ^r	Staskawicz <i>et al.</i> (1987)
pRK415	Broad-host-range cloning vector; Tc ^r	Keen <i>et al.</i> (1988)
pRK2013	Broad-host-range (ColE1 Tra _{RP1}) helper vector; Km ^r	Figurski & Helinski (1979)
pHP45ΩHg	Derivative of pHP45:Ω where the Sm ^r /Sp ^r of the Ω interposon is replaced by the HgCl ₂ resistance operon of Tn501	Fellay <i>et al.</i> (1987)
pUCP18/19	<i>E. coli</i> - <i>P. aeruginosa</i> shuttle cloning vector; Ap ^r /Cb ^r	Schweizer (1991a)
pMP190	Broad-host-range, low-copy-number <i>lacZ</i> transcriptional fusion vector; Cm ^r Sm ^r	Spaink <i>et al.</i> (1987)
pSUP202	pBR325 derivative carrying the Mob (mobilization) site of plasmid RP4; gene replacement vector; Ap ^r /Cb ^r , Tc ^r , Cm ^r	Simon <i>et al.</i> (1983)
pSUP202ΔTc	Derivative of pSUP202 carrying a deletion within the <i>tet</i> gene removing the unique <i>Bam</i> HI and <i>Hind</i> III sites in this vector; Ap ^r /Cb ^r , Cm ^r	Dean & Poole (1993b)
pPZ30	Broad-host-range <i>lacZ</i> translational fusion vector; Ap ^r /Cb ^r	Schweizer (1991b)
pEX100T	pUC19-based gene replacement vector; Ap ^r	Schweizer & Hoang (1995)
pTON-12	pSUP202ΔTc:: <i>tonB::ΩHg</i>	This study
pTON-13	pEX100T:: <i>tonB::ΩHg</i>	This study

* Ap^r, ampicillin-resistant; Cb^r, carbenicillin-resistant; Cm^r, chloramphenicol-resistant; Sm^r, streptomycin-resistant; Sp^r, spectinomycin-resistant; Tc^r, tetracycline-resistant; *tonB::ΩHg*, *tonB* gene disrupted by insertion of the ΩHg interposon.

were recovered, dialysed to remove the sucrose and ligated to the broad-host-range cosmid cloning vector pLAFR3 previously treated with *Bam*HI and calf intestinal alkaline phosphatase (Sambrook *et al.*, 1989). Following *in vitro* packaging of the ligated DNA using the Packagene Lambda DNA Packaging System (Promega) and infection of *E. coli* HB101, cosmid-containing HB101 clones were recovered on tetracycline-containing L-agar. Approximately 2000 individual colonies were obtained and analysis of the cosmids from 18 randomly selected colonies revealed a variety of inserts of *ca* 15–25 kb in each case. The colonies were recovered in batches of 10, cultured overnight in L-broth containing tetracycline and stored at –20 °C.

Cloning of the *tonB_{pa}* gene. The pLAFR3 library of *P. aeruginosa* chromosomal DNA was mobilized *en masse* into the *P. putida tonB* strain TE156 using pRK2013 and a triparental

mating procedure (see above). Following the overnight incubation, however, bacterial growth was resuspended in 1 ml of iron-deficient (succinate) minimal medium and washed three times in the same medium before being plated onto iron-deficient (succinate) minimal medium plates containing tetracycline and nalidixic acid. Cosmid DNA was subsequently prepared from colonies appearing after 48 h incubation at 30 °C and re-introduced into TE156 via triparental mating to confirm the ability of the cosmid DNA to facilitate growth of TE156 on iron-deficient minimal medium.

Nucleotide sequencing. DNA for sequencing was obtained as double-stranded template and sequenced by the Centres of Excellence Core Facility for Protein/DNA Chemistry at Queen's University. Overlapping sequence from both strands was obtained using a series of custom-synthesized primers. Because of difficulties in cloning the 2.8 kb *tonB*-containing *Pst*I

Fig. 1. Subcloning of the *P. aeruginosa* *tonB* gene and generation of *tonB-lacZ* transcriptional/translational fusions. The indicated restriction fragments were cloned into vector pRK415 (pTON-1 to -5) and introduced into *P. putida* TE156 where they were assessed for an ability to restore growth on an iron-limited minimal medium (+, growth; -, no growth). The *Bam*HI site of insertion of an Ω Hg interposon is indicated, as is the position and orientation of the *tonB*_{P.a.} gene. The 5' region of *tonB*_{P.a.} and upstream sequences were isolated on a *Bam*HI fragment or a *Msc*I-*Bsr*BI fragment from pTON-4 and cloned, respectively, into the *lacZ* transcriptional fusion vector pMP190 (to generate pTON-6) and the *lacZ* translational fusion vector pPZ30 (to generate pTON-11). *Bam*HI* indicates a *Bam*HI site in the pRK415 multicloning site. *Msc*I* indicates a non-unique *Msc*I site from pTON-4.

fragment of pTON-4 onto sequencing vectors such as pUCP18/19, this region was actually cloned as two separate *Bam*HI-*Pst*I fragments in pUCP18 and sequenced. A 500 bp *Msc*I fragment which encompasses the *Bam*HI site in *tonB* was also cloned in pUCP18 and sequenced to provide sequence data across this restriction site. Nucleotide and deduced amino acid sequences were analysed using the PC Gene software package (Intelligenetics).

Expression of the cloned *tonB*_{P.a.} gene. To identify the product of the cloned *tonB*_{P.a.} gene, the phage T7-based expression system of Tabor & Richardson (1985) was employed as described previously (Poole *et al.*, 1993a).

In vitro mutagenesis of *tonB*_{P.a.} The cloned *tonB*_{P.a.} gene was mutagenized *in vitro* by the insertion of an HgCl₂ resistance interposon (Ω Hg) into the unique *Bam*HI site in *tonB*_{P.a.}. Briefly, the *tonB*_{P.a.}-containing 2.8 kb *Pst*I fragment of plasmid pTON-4 was cloned into pSUP202 Δ Tc, a derivative of the gene replacement vector pSUP202 carrying a deletion in the *tet* gene which eliminates the previously unique *Bam*HI and *Hind*III sites in this vector. This recombinant (pTON-12) was subsequently digested with *Bam*HI and ligated to a 5 kb *Bam*HI fragment of plasmid pHP45 Ω Hg carrying the Ω Hg interposon. The *tonB*:: Ω Hg construct was then recovered on a 7.8 kb *Pst*I fragment and cloned into the unique *Pst*I site of pRK415 to yield pTON-5 (Fig. 1).

Construction of a *P. aeruginosa* *tonB* mutant. Introduction of pTON-12 into *E. coli* S17-1 and mobilization into *P. aeruginosa* via conjugation, according to a previously described procedure

(Poole *et al.*, 1993a), failed to generate the desired *tonB*:: Ω Hg strains of *P. aeruginosa*. Thus, the *tonB*:: Ω Hg-carrying 7.8 kb *Pst*I fragment (see above) was cloned into the unique *Sma*I site of the gene replacement vector pEX100T following conversion of this site to a *Pst*I site with *Pst*I linkers (Stratagene) according to the manufacturer's instructions. The recombinant pEX100T carrying the Ω Hg-disrupted *tonB* gene (called pTON-13) was subsequently transformed into *E. coli* S17-1 and mobilized into *P. aeruginosa* via conjugation as described by Poole *et al.* (1993a). To permit counterselection of the donor *E. coli* strain in these experiments, the recipient *P. aeruginosa* strains were either streptomycin-resistant or spontaneous streptomycin-resistant derivatives were isolated as described above. Following conjugation, the mating mixture was plated on L-agar containing streptomycin, HgCl₂ and sucrose (5%, w/v) and colonies appearing after 48–72 h incubation at 37 °C were recovered. The *tonB* genes were amplified (by PCR) from representative colonies of each mating to confirm disruption of the chromosomal *tonB* gene by replacement with the *tonB*:: Ω Hg construct. Briefly, chromosomal DNA was prepared as described by Ausubel *et al.* (1992) from *P. aeruginosa* *tonB*:: Ω Hg candidates and amplified using *Taq* DNA polymerase (Life Technologies) and primers *tonB*1 (5' CCTGCCATGCGTGAATGC 3') beginning at position 203 of the sequence presented in Fig. 1 and *tonB*2 (5' AGAACATCTTGGTCGCTGG 3') complementary to and beginning at position 1108 of the Fig. 1 sequence. The reaction mixture (100 μ l) contained 2.5 U *Taq* DNA polymerase, 0.5 μ M each primer, 0.2 mM each deoxynucleoside triphosphate, 1.5 mM MgCl₂, 10% (v/v) dimethylsulphoxide, 100 ng genomic DNA and 1 $\times$ PCR buffer (Life Technologies). The mixture was treated for 2 min at 94 °C followed by 35 cycles of 45 s at 94 °C, 1 min at 52 °C and 2 min at 72 °C, before finishing with 10 min at 72 °C. PCR products were examined on 0.8% (w/v) agarose gels.

Growth assays of *P. aeruginosa* *tonB* mutants. *tonB* mutants and their parent strains were cultured at 37 °C overnight in BHI broth and subsequently diluted into BHI broth in the presence and absence of ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDHA) (25 μ g ml⁻¹ for PAO6609 and its *tonB* derivative K883; 10 μ g ml⁻¹ for K372 and its *tonB* derivative K886) to a final OD₆₀₀ of 0.05–0.1. Bacterial growth was assayed by monitoring the change in OD₆₀₀ over time of cultures shaken (200 r.p.m.) at 37 °C. To assess the ability of *tonB* mutants and their parents to utilize siderophores, 1 ml iron-deficient (succinate) minimal medium containing EDDHA (150 μ g ml⁻¹), with and without siderophore supplementation [pyoverdine, 100 μ g ml⁻¹; enterobactin, 50 μ l concentrated *E. coli* culture supernatant ml⁻¹ (see below)], was inoculated with 10⁷ organisms, incubated overnight at 37 °C and assessed visually for growth.

Growth promotion by pyoverdine and pseudobactin. The *P. putida* WCS358 *tonB* strain TE156 (10⁷ organisms) was plated onto the surface of an iron-deficient (succinate) minimal plate and filter discs impregnated with various concentrations of pyoverdine_{PAO} (prepared from *P. aeruginosa* PAO1) and pseudobactin₃₅₈ (prepared from *P. putida* WCS358) placed on the plates. Following overnight incubation at 30 °C, plates were examined for evidence of growth in the region surrounding the filter discs.

Growth promotion by enterobactin. Enterobactin-containing 25-fold concentrated spent culture supernatant was prepared from 1 litre overnight cultures of *E. coli* JR20 as described previously (Poole *et al.*, 1990). To assess the ability of this enterobactin to stimulate the growth of selected *E. coli* strains and their plasmid-containing derivatives, strains were cultured

overnight in iron-deficient (glucose) minimal medium supplemented with arginine and methionine and diluted to an OD₆₀₀ of 0.05 in the same medium containing EDDHA (100 µg ml⁻¹) and enterobactin (7.5 µl concentrated spent culture supernatant ml⁻¹). Cultures were incubated at 37 °C with shaking and growth (change in OD₆₀₀) monitored over time. Plate assays of enterobactin growth promotion were carried out by plating 10⁷ organisms onto iron-deficient (glucose) minimal medium plates containing arginine, methionine and EDDHA (see above) and overlaying filter discs impregnated with varying concentrations of enterobactin (as concentrated culture supernatant). Following overnight incubation at 37 °C, plates were examined for evidence of growth surrounding the filter discs.

Growth promotion by vitamin B₁₂. To assess the ability of vitamin B₁₂ to promote the growth of selected *E. coli metE* strains and their plasmid-containing derivatives, these strains were cultured overnight in iron-deficient minimal (glucose) medium supplemented with vitamin B₁₂ (5 nM) and diluted to OD₆₀₀ = 0.05 in the same medium. Cultures were then incubated at 37 °C with shaking and growth monitored as described above. Plate assays of vitamin B₁₂ growth promotion were carried out as described above except that the plates were not supplemented and the discs were impregnated with varying concentrations of vitamin B₁₂.

Plate assay for phage sensitivity. To assess the sensitivity of various *E. coli* strains to phage φ80, 5 µl 10-fold dilutions of a phage stock were spotted onto L-agar plates streaked with 10⁷ bacteria. Plaque formation was assessed following overnight incubation at 37 °C.

β-Galactosidase assays. *P. aeruginosa* carrying derivatives of the *lacZ* fusion vector pMP190 were cultured overnight at 37 °C in BHI containing chloramphenicol and diluted 100-fold into fresh BHI without antibiotic. Following growth to early exponential phase, EDDHA was added, where indicated, and all cultures incubated a further 1.5 h before being assayed for β-galactosidase activity as described by Miller (1992).

RESULTS

Cloning of *tonB*_{P.a.}

To clone *tonB*_{P.a.}, attempts were made to complement the growth defect of a *P. putida tonB* strain (TE156) on iron-deficient minimal medium. Thus, a pLAFR3 library of *P. aeruginosa* PAO DNA was moved into *P. putida* TE156 and colonies growing on iron-deficient minimal medium recovered. Cosmid DNA isolated from these colonies exhibited a common 8.5 kb *EcoRI* fragment which itself was able to restore growth of TE156 on iron-deficient minimal medium (Fig. 1; plasmid pTON-1). The fragment also restored the ability of TE156 to utilize iron-siderophore complexes as confirmed by pyoverdine- and pseudobactin-dependent growth of pTON-1-containing TE156 (but not TE156 itself) on EDDHA-containing minimal medium (data not shown). Cleavage at the unique *Bam*HI site on the *EcoRI* fragment (plasmids pTON-2 and pTON-3) abolished complementation of the growth defect (Fig. 1), suggesting that this restriction site occurs within the complementing gene. Indeed, a 2.8 kb *Pst*I fragment encompassing the *Bam*HI site (pTON-4) restored growth of TE156 on iron-deficient medium and insertion of ΩHg into the *Bam*HI site (pTON-5) abolished growth restoration (Fig. 1).

	-35	-10	
GGTCGCGGAATGATCCGCCAAGGTTGCCCTGAGGACGCGACTGAAT		GATAATAATATCA	60
		<i>S. D.</i> <i>tonB</i>	
GAAGAGCTCTACTTCTTACCTTCTCTCCGAGGTCGCCCTCATGTGCCACAGCCT		M S P Q P	120
TCACGGTCGCTGACCGTTTTTCGCTGGCCGCGCTCGCCGAAGACCACCCACGCTCCC		<i>Bsr</i> BI	180
S R S P D R F S L A A L A E D H P T A P			
GCCGAGGAGACGAAAGCGAGTCCCTGCCATGCGTGAATGCCAGCGCGGAGAGCCCAAC			240
A Q G D E S E S L P C V N A Q R G E P N			
CTGCGGTGGTGGACTGCTCCGTCGCGCGCGACGAGGAAGTGGCGTGCAGGAAGTG			300
L R V V D C S G A R R D E E V A V E E V			
CTGATCCCCACGCCACGCGAGCGATCCCAAGACGTCGCCGCGAGCCGCCCAAGTCG			360
L I P Y A H G S D P E D V P G E P P K S			
CGCTGGTGGTGTCTCCGCGCAGCGGTGGCGATGCATGTGGCGATCATCGTGGCGTG			420
R W W L S S G A A V A M H V A I I G A L			
GTGTGGTGTGTCGCGACCCCGCGCAACTCAACCTTGCCACGCGCAACTGCCGAAGACC			480
V W V M P T P A E L N L G H G E L P K T			
ATGACGTTGAATTCGTCGAGCTCGAGAAGAGGCCGACGCCGCCGCGCCGCGCT			540
M Q V N F V Q L E K K A E P T P Q P P A			
GCCGCGCGGAAACCGACGCCGCCGAAGATCGAGGAACCAAGCCGAGCGCGCAAGCGC			600
A A P E P T P P K I E E P K P E P P K P			
AAGCGGTGGAACCAACCAAGCCGCAAGCCGCAAGCCGCAAGCCGCGTGGAAACCGCC			660
K P V E K P K P K P K P K P V E N A			
ATTCCCAAGGCCAAGCCGAGCCGCAACCAACCAAGCCGCAAGCCGAGCCGAGCAGC			720
I P K A K P K P E P K P K P E P E P S T			
GAAGCATCCAGCCAGCCGCCGCTCCAGCGCGCGCGCGCGCGCGCTACCGTCGGG			780
E A S S Q P S P S S A A P P P A P T V G			
CAGAGCAGCCCGCGCGCAGACTGCGCCAGCGGCTCGCAAGGCCCGCGCGCGCTGCC			840
Q S T P G A Q T A P S G S Q G P A G L P			
AGCGGCGAGCTCAACGACGCGACATCAAGCCGCTGCGCATGGATCTCCGGTCTATCCG		<i>Bam</i> HI	900
S G S L N D S D I K P L R M D P P V Y P			
CGGATGGCCGAGCGCGCGGTATCGAAGGCCGGTGAAGGTGCTGTCCACCATCACCAGC			960
R M A Q A R R I E G R V K V L F T I T S			
GATGGCGCATTGACGACATCCAGGTGTGAGTGGTGGCGTGCCTGCGCATGTTCCAGCCG			1020
D G R I D D I Q V L E S V P S R M F D R			
GAAGTGCAGCCAGCCATGGCCAAGTGGCGTTTCGAGCCGCGTGTGAGCGGTGGCAAGATC			1080
E V R Q A M A K W R F E P R V S G G K I			
GTCGCCGCCAGCGGACCAAGATGTTCTTCTTCAAGATCGAGAAGCGCGCTGAGGCGGT			1140
V A R Q A T K M F F F K I E K R R			
TCGCCGACAAAGAGCGCGCTTCGAGCGCGGTTTTTTATCCCGTAGGTCTC			1200

Fig. 2. Nucleotide sequence and translation of the *tonB*_{P.a.} gene. Putative promoter (−35, −10) and Shine–Dalgarno (S.D.) sequences are indicated, as is a sequence exhibiting homology to the consensus Fur binding sequence (underlined). The *Bam*HI site of insertion of an ΩHg interposon in plasmid pTON-5 (Fig. 1) and the *Bsr*BI site of in-frame fusion to *lacZ* in plasmid pTON-11 (Fig. 1) are also indicated. An internal signal-like sequence in the translated TonB product is italicized.

Nucleotide sequence of *tonB*_{P.a.}

To identify the gene(s) complementing the growth defect in the *P. putida tonB* strain TE156, the region of pTON-4 flanking the *Bam*HI site was sequenced. The largest ORF identified (1025 bp) begins with an ATG start codon and was designated *tonB*_{P.a.} (Fig. 2). Capable of encoding a polypeptide of 341 amino acids (minus the initiation methionine) with a molecular mass of 36860 Da, *tonB*_{P.a.} directed the expression [on plasmid pT7-6 (Tabor & Richardson, 1985) in *E. coli*] of a protein of 51 kDa (data

Table 2. Iron-regulated β -galactosidase activity of *P. aeruginosa* carrying *tonB-lacZ* transcriptional and translational fusions

P. aeruginosa PAO6609 carrying a *tonB-lacZ* transcriptional (pTON-6) or translational (pTON-11) fusion was cultured overnight in BHI broth containing the appropriate antibiotic and subcultured into the same medium supplemented with the indicated concentrations of EDDHA. Following growth to exponential phase, the cultures were assayed for β -galactosidase activity as described in Methods.

Vector	EDDHA ($\mu\text{g ml}^{-1}$)	β -Galactosidase activity (Miller units)*
pTON-6	0	488 $\pm$ 41
	25	2201 $\pm$ 74
pTON-11	0	6.5 $\pm$ 1.0
	25	95.0 $\pm$ 1.2

* The data represent the mean of three determinations $\pm$ SD.

not shown). Although additional candidate translation initiation sites (GTG) exist within the *tonB*_{P.a.} ORF, in-frame fusion of *tonB*_{P.a.} sequences upstream of a *Bsr*BI restriction site (includes the ATG codon but none of the GTG codons) (Fig. 2) to *lacZ* in the translational fusion vector pPZ30 (Fig. 1; pTON-11) yielded active enzyme (Table 2), suggesting that translation initiation begins upstream of this restriction site, likely at the indicated ATG. Expression of this translational fusion as well as a transcriptional *tonB*_{P.a.}-*lacZ* fusion (Fig. 1; pTON-6) was markedly enhanced under iron-limiting (+ EDDHA) conditions (Table 1). Consistent with this, a sequence exhibiting homology to the consensus Fur binding sequence (13/19 matches) was identified upstream of *tonB*_{P.a.}, overlapping a putative σ^{70} promoter region (Fig. 2).

The deduced TonB_{P.a.} polypeptide is rich in Pro (18.4%) and exhibits a (Lys-Pro)₆ repeat typical of previously described TonB proteins. Although a substantial Glu-Pro repeat sequence, also typical of TonB proteins, was absent from the Pro-rich domain of the TonB_{P.a.} homologue, this region did exhibit some short Glu-Pro sequences (Fig. 2). A hydropathy plot of the deduced TonB sequence revealed a single region of substantial hydropathicity (L88-M108) (Fig. 2) predicted to be membrane-spanning (data not shown). This region is preceded by two basic amino acids (K83 and R85), suggestive of a leader/signal sequence, and may, in fact, represent an internal signal/membrane anchor sequence.

Isolation and characterization of a *tonB* mutant strain of *P. aeruginosa*

Despite many previous successes using the gene replacement vector pSUP202 and its relatives to construct knockout mutations in various *P. aeruginosa* genes (Poole

Fig. 3. Growth of *P. aeruginosa* strain PAO6609 (●, ○) and the *tonB* derivative K883 (■, □) in BHI broth under iron-rich (-EDDHA) (●, ■) and iron-limited (+EDDHA) (○, □) conditions.

et al., 1993a, b, c; Heinrichs & Poole, 1993; Dean & Poole, 1993b) all attempts at constructing a *tonB* mutant using pTON-12 (pSUP202ΔTc::tonB::ΩHg) were unsuccessful. Thus, the ΩHg-disrupted *tonB* gene was cloned into the newly described gene replacement vector pEX100T and ultimately inserted into the *P. aeruginosa* chromosome, replacing the wild-type copy of the gene. Successful gene replacement (as confirmed by PCR analysis of the chromosomal *tonB* genes, data not shown) was achieved in the pyoverdine-deficient strain PAO6609 (yielding strain K883) and the pyoverdine-/pyochelin-deficient strain K372 (yielding strain K886). *tonB* mutants of the pyoverdine-producing PAO1 derivative K335 were, however, not obtained, despite numerous attempts. As reported previously for *tonB* strains of other bacteria (Bitter *et al.*, 1993; Wang & Newton, 1969), the *tonB* mutants K883 (Fig. 3) and K886 (data not shown) grew poorly in an iron-limited (+ EDDHA) but not iron-rich medium while the parent strains PAO6609 (Fig. 3) and K372 (data not shown) exhibited no such growth defect in an iron-limited medium. Moreover, the growth restriction imposed on pyoverdine-deficient strains by 150 $\mu\text{g EDDHA ml}^{-1}$ in minimal medium (Poole *et al.*, 1991), while reversed by the exogenous addition of pyoverdine or enterobactin in the case of PAO6609 and K372, was not reversed by these siderophores in the *tonB* mutants (data not shown).

Homology of TonB_{P.a.} and other TonB proteins

Although the *tonB*_{P.a.} gene was cloned by its ability to complement certain features of the *tonB* mutation in *P. putida* TE156, the predicted TonB_{P.a.} product was not very similar to the *P. putida* TonB protein (22.3% identity). Indeed, other than the Pro-rich domain, no regions of substantial homology were identifiable in these proteins (data not shown). In contrast, TonB_{P.a.} showed

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TonBP.a. SPQPSRSPDRFSLAALAEHPTAPAGQDESESLPCVNAQRGEPNLRVDC 50
TonBE.c. -----
TonBP.a. SGARRDEEVAVEEVLIPYAHGSDPEDVPGEPPKSRNWLSGGAAMHVAI 100
TonBE.c. -----TLDLPRRFPW--PTLLSVCIHGAV 22
TonBP.a. IGALVWVMPAPAEINLGHGELPKTMQVNFVLEKKAEPPOPPAAAEPT 150
TonBE.c. VAGLLYTSVHQV-----IELPAPAQIPSVMTVT 50
TonBP.a. PPKIEEPKPEPPKPKPKVEKPKPKPKPKPV-ENAIKPKPKPEPKPKPE 199
TonBE.c. PADLEPPQAVQPPPEPVVEPEPEPEPIPEPPKEAPVVIKPKPKPKPKPK 100
TonBP.a. PEPSTEASSQSPSSAAPPAPTAVGQSTPGAQTPAGSGSGPAGLPSGSLN 249
TonBE.c. PVKKVQEOPKRDVQKPVESRPAFPFENTAPARLTSSSTATAATSKPVTSV-- 148
TonBP.a. DSDIKPLRMDPPVYPRMAQARRIEGRVKVLETITSDGRIDDIQVLESVPS 299
TonBE.c. ASGPRALSRNOPQYPARAOLRIEGQVKVKEDVTPDGRVDNVQILSAKPA 198
TonBP.a. RMEDREVRQAMAKWREPRVSGGKIVARQATKMFFFKIE---KRR 341
TonBE.c. NMFEREVKNAMRRRYEPGKPGSGIVVN-----ILFKINGTTEIQ 238

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Fig. 4. Alignment of the deduced amino acid sequences of the TonB proteins of *P. aeruginosa* and *E. coli*. The alignment was carried out using the PALIGN program of the PC Gene software package (Intelligenetics). Exact matches (|) and conserved changes (.) are indicated. The cytoplasmic membrane-associated domain of TonB_{E.c.} and a predicted transmembrane region of TonB_{P.a.} are italicized. The latter was identified using the PC Gene SOAP program. A region conserved in the TonB proteins of *P. aeruginosa*, *E. coli* (Postle & Good, 1983; Postle & Skare, 1988), *Sal. typhimurium* (Hannavy *et al.*, 1990), *Ent. aerogenes* (Bruske & Heller, 1993), *K. pneumoniae* (Bruske *et al.*, 1993), *Serr. marcescens* (Gaisser & Braun, 1991) and *Y. enterocolitica* (Koebnik *et al.*, 1993) is underlined.

substantially more homology to TonB_{E.c.} (33.2% identity). In addition to the Glu-Pro/Lys-Pro domain, which is reasonably conserved between these two proteins, a region near the C terminus of TonB_{P.a.} was highly similar to a region at the C terminus of TonB_{E.c.} (Fig. 4). This C-terminal domain is also conserved in the majority of TonB proteins so far sequenced (Fig. 4), the exceptions being the TonB proteins of *P. putida* and *H. influenzae*. Moreover, residues Gly-174, Gly-186 and Arg-204 of TonB_{E.c.}, shown to be important for certain activities of this protein (Traub *et al.*, 1993), are conserved in these TonB proteins (Fig. 4). TonB_{P.a.} is substantially larger than previously described TonB proteins (341 residues in TonB_{P.a.} vs. 239–261 residues in the other TonB proteins).

Activity of TonB_{P.a.} in *E. coli*

Given the homology between TonB_{P.a.} and TonB_{E.c.}, it was of interest to see if TonB_{P.a.} could functionally replace TonB_{E.c.} in a *tonB* mutant strain. Thus, the cloned *tonB_{P.a.}* gene (on plasmid pTON-4) was introduced into the *tonB metE* strain RK5015 and tested for its ability to restore a number of TonB-dependent functions, including utilization of ferric enterobactin and vitamin B₁₂, as well as sensitivity to phage ϕ 80. The methionine requirement of RK5015 can be met by vitamin B₁₂. However, this

requires TonB-dependent transport of vitamin B₁₂ into the cell. Thus, RK5015 failed to grow in minimal medium or on minimal medium plates when supplemented with vitamin B₁₂ while its TonB⁺ parent strain RK5173 grew well under these conditions with a generation time in liquid medium of 0.91 h. RK5015 carrying pTON-4 was able to grow on minimal medium containing vitamin B₁₂, although less well than RK5173 (generation time = 2.08 h), indicating that TonB_{P.a.} is active in *E. coli*. Similarly, RK5015 failed to grow on EDDHA-containing minimal medium despite enterobactin supplementation while RK5173 alone and RK5015 carrying pTON-4 demonstrated enterobactin-dependent growth in this medium, with generation times in liquid medium of 1.75 and 7.34 h, respectively. Finally, sensitivity to phage ϕ 80, absent in RK5015, was restored by the *tonB_{P.a.}* gene, although the plaques formed on RK5015/pTON-4 were quite hazy compared to those observed on RK5173, which were extremely clear and well-defined (data not shown). Nonetheless, the numbers of p.f.u. enumerated at any given phage dilution was essentially the same for both strains (data not shown).

DISCUSSION

The identification, in a variety of bacteria of TonB homologues (Bitter *et al.*, 1993; Bruske & Heller, 1993; Bruske *et al.*, 1993; Hannavy *et al.*, 1990; Karlsson *et al.*, 1993b; Klesney-Tait *et al.*, 1994; Koebnik *et al.*, 1993; Jarosik *et al.*, 1994) and outer-membrane proteins exhibiting homology to the family of TonB-dependent receptors (Braun *et al.*, 1987; Cornelissson *et al.*, 1992; Heller & Kadner, 1985; Henderson & Payne, 1994; Jarosik *et al.*, 1994, 1995; Stojiljkovic & Hantke, 1992) would seem to indicate that the TonB mechanism of energy-/receptor-dependent ligand uptake across the Gram-negative outer membrane (Postle, 1990a, 1993) is well-conserved in prokaryotes. TonB_{P.a.} displays a moderate level of homology (30% identity, or 40% identity if the additional N-terminal residues of TonB_{P.a.} are excluded) to TonB_{E.c.} and is able to, albeit weakly, restore a number of TonB-dependent functions in an *E. coli tonB* mutant. This means, presumably, that TonB_{P.a.} is able to interact, in *E. coli*, both with outer-membrane receptor proteins and auxiliary proteins (e.g. ExbB, ExbD) required for TonB activity and, moreover, that residues conserved between TonB_{P.a.} and TonB_{E.c.} include those important for these interactions.

While there is limited homology between the N-terminus of TonB_{E.c.}, a region implicated in the interaction with ExbB in *E. coli* (Larsen *et al.*, 1994; Karlsson *et al.*, 1993a; Jaskula *et al.*, 1994; Traub *et al.*, 1993), and the corresponding region of TonB_{P.a.}, this region of both proteins is characterized by the presence of a hydrophobic, predicted (in the case of TonB_{P.a.}) membrane-spanning α -helical domain. Furthermore, His-20 of TonB_{E.c.} (His-19 in the mature protein is depicted in Fig. 4), which appears to be very important for TonB activity (Traub *et al.*, 1993), is conserved in TonB_{P.a.}. These may explain, in part, the activity of TonB_{P.a.} in *E. coli* and are probably

important for the interaction with ExbB in *E. coli* and, possibly, in *P. aeruginosa*.

The Glu-160 residue of TonB_{E.c.}, implicated in receptor interaction (Heller *et al.*, 1988; Bell *et al.*, 1990) and similarly important for TonB_{E.c.} activity (Traub *et al.*, 1993; Bruske & Heller, 1993), is not, however, conserved (at least at the same position) in TonB_{P.a.}. It is possible, nonetheless, that in the context of the TonB_{P.a.} protein, some other residue fulfills this role in receptor interaction. Despite the lack of an obvious Glu-160 counterpart in TonB_{P.a.}, a region immediately downstream of this residue is highly homologous between the two proteins and fairly well-conserved amongst most TonB proteins. This domain overlaps a region capable of forming an amphipathic α -helix which has been implicated in receptor interaction (Larsen *et al.*, 1993). Moreover, several mutants exhibiting altered TonB activity in *E. coli* have been mapped to this region of TonB_{E.c.} (Traub *et al.*, 1993). This region may, therefore, be important for the function of TonB_{P.a.} both in *E. coli* and in *P. aeruginosa*.

Although the apparent molecular mass of TonB_{P.a.} is substantially larger than that predicted from the *tonB*_{P.a.} sequence, anomalous migration on SDS-polyacrylamide gels is a hallmark of TonB proteins, which typically exhibit apparent molecular masses some 10 kDa larger than that predicted from gene sequences [e.g. TonB_{E.c.} is predicted to be 26 kDa based on nucleotide sequence data but migrates on polyacrylamide gels with an apparent molecular mass of 36 kDa (Postle & Good, 1983)]. The larger apparent molecular mass of TonB_{P.a.} relative to TonB_{E.c.} is, however, consistent with the sequence data which predicts it to be larger.

The alignment presented in Fig. 4 indicates that the bulk of the additional residues in TonB_{P.a.} occurs at the N terminus of the protein, upstream of the predicted membrane-spanning, putative internal signal/membrane anchor sequence. As such, it is likely that the extra residues are disposed to the cytoplasmic side of the cytoplasmic membrane. The functional significance of this novel N-terminal extension is unknown and no homology between this domain and known proteins was identified during a search of the GenBank databases. It is intriguing to note, however, that an N-terminal extension was previously identified in the ExbB homologue of *P. putida* (ExbB_{P.p.}) (Bitter *et al.*, 1993) which was, nonetheless, active in *E. coli*. It was speculated that the presence of this N-terminal domain in ExbB_{P.p.} is somehow related to the nature of some TonB-dependent ferric siderophore receptors in this organism which are characterized by N-terminal, presumed periplasmic, extensions not typical of most TonB-dependent receptors (Bitter *et al.*, 1993). Interestingly, the outer-membrane receptor for ferric pyoverdine in *P. aeruginosa*, FpvA, a protein showing homology to the TonB-dependent family of receptors, also appears to possess a periplasmic N-terminal extension (Poole *et al.*, 1993c). It is possible, therefore, that the presence of this periplasmic domain in these outer-membrane receptors necessitates changes in the TonB-ExbB-ExbD complexes of these organisms

which might explain the additional N-terminal domains in ExbB_{P.p.} and TonB_{P.a.}.

ACKNOWLEDGEMENTS

We thank Jan Tommassen and Robert Kadner for supplying strains and Peter Weisbeek and Jean-Marie Meyer for providing pseudobactin and pyoverdine, respectively. This work was supported by a grant from the Medical Research Council of Canada. K. P. is an NSERC University Research Fellow. D. H. is the recipient of a Canadian Cystic Fibrosis Foundation studentship. C. D. is an Ontario Graduate Scholar.

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Received 12 January 1996; accepted 22 January 1996.