Disruption of *tonB* in *Bordetella bronchiseptica* and *Bordetella pertussis* prevents utilization of ferric siderophores, haemin and haemoglobin as iron sources

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The *Bordetella bronchiseptica tonB* gene was cloned by detection of a chromosomal restriction fragment hybridizing with each of two degenerate oligonucleotides that corresponded to Pro-Glu and Pro-Lys repeats characteristic of known TonB proteins. The *tonB_{bb}* gene was situated upstream of *exbB* and *exbD* homologues and downstream of a putative Fur-regulated promoter. Hybridization results indicated that the *tonB* operon and flanking regions were highly conserved between *B. bronchiseptica*, *Bordetella pertussis* and *Bordetella parapertussis*. Disruption of *tonB* in *B. bronchiseptica* resulted in inability to grow in iron-limiting media, and inability to utilize alcaligin, enterobactin, ferrichrome, desferroxamine B, haemin and haemoglobin. Although it was not possible to inactivate *tonB* in a clinical *B. pertussis* isolate, *tonB* was disrupted in a laboratory *B. pertussis* strain previously selected for the ability to grow on Luria–Bertani medium. This *B. pertussis tonB* mutant shared a similar iron complex utilization deficient phenotype with the *B. bronchiseptica tonB* mutant. The *B. bronchiseptica tonB* operon present on a plasmid did not complement an *Escherichia coli tonB* mutant, but inefficient reconstitution of enterobactin utilization was observed in one *fepA* mutant harbouring plasmid copies of the *B. pertussis fepA* homologue and *tonB_{bb}* operon.

Keywords: *Bordetella*, iron sources, TonB-dependent receptors

INTRODUCTION

The acquisition of iron-siderophores, haem/haemin, transferrin, vitamin B₁₂ and certain colicins in various Gram-negative bacteria is mediated through an energy-coupled process, in which outer-membrane receptors and the cytoplasmic membrane proteins TonB, ExbB and ExbD are involved (Klebba *et al.*, 1993; Postle, 1993; Braun, 1995). It is likely that TonB-dependent iron complex uptake in mammalian hosts is of key importance for Gram-negative pathogens. For example, a *Haemophilus influenzae tonB* mutant proved to be

avirulent in a rat model (Jarosik *et al.*, 1994), and a *Vibrio cholerae tonB* mutant failed to cause disease in infant mice (Henderson & Payne, 1994).

In current models for TonB-dependent energy transduction, the proline-rich TonB protein is anchored to the cytoplasmic membrane by its N-terminus while the remainder of the protein occupies the periplasmic space and makes contact with outer-membrane receptors (Klebba *et al.*, 1993; Postle, 1993; Braun, 1995). Contact by TonB with outer-membrane receptors is believed to induce a conformational change of the receptors, resulting in transport of bound ligands into the periplasmic space (Skare *et al.*, 1993). Since ligand translocation into the periplasm is energy dependent, TonB is believed to transduce the cytoplasmic membrane proton-motive force to the receptors.

In order to function efficiently, TonB requires the two

Abbreviations: DP, 2,2'-dipyridyl; EDDA, ethylenediamine-di-(o-hydroxy-phenylacetic acid); LB, Luria–Bertani; SS, Stainer–Scholte minimal broth lacking added iron; SS-EDDA, SS agar containing 45 µg EDDA ml⁻¹.

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auxiliary proteins, ExbB and ExbD. Most of ExbB is situated cytoplasmically, and it is predicted to span the cytoplasmic membrane three times, with a short N-terminal region and a short turn situated in the periplasm (Kampfenkel & Braun, 1993). Like TonB, ExbD is predicted to have a short N-terminal region anchored in the cytoplasmic membrane, with most of the protein situated in the periplasm (Kampfenkel & Braun, 1992). ExbB and ExbD interact with and activate TonB, and it is likely that the three proteins form a complex (Fischer *et al.*, 1989b; Skare & Postle, 1991; Ahmer *et al.*, 1995; Braun *et al.*, 1996).

The important mammalian respiratory pathogens *Bordetella bronchiseptica* and *B. pertussis* express outer-membrane receptors that are regulated by iron availability and are believed to be involved in the uptake of iron complexes. One such receptor is BfeA, a FepA homologue, that has been shown to be required for utilization of the exogenous siderophore enterobactin (Beall & Sanden, 1995b). More recently, the iron-regulated outer-membrane proteins BfrA, BfrB and BfrC have been identified (Beall & Hoenes, 1997; Beall, 1998). BfrA is specific to *B. bronchiseptica*, whereas BfrB, BfrC and BfeA appear to be highly conserved among *Bordetella* species. All four *Bordetella* proteins belong to the family of TonB-dependent outer-membrane receptors, based on their high level of amino acid sequence identity to many other bacterial proteins belonging to this group. Also, all four genes are Fur-repressed, induced under low-iron conditions, and contain consensus Fur-binding sequences in their promoter regions (Beall & Sanden, 1995a, b; Beall & Hoenes, 1997; Beall, 1998; Brickman & Armstrong, 1995). The ligand specificities of BfrA, BfrB and BfrC are still unknown.

Presumably, *Bordetella* species also have a specific receptor for ferric complexes of the *Bordetella* siderophore, alcaligin (Moore *et al.*, 1995; Brickman & Armstrong, 1996a) and for complexes of exogenously supplied ferrichrome, desferrioxamine B and haemin (Beall & Hoenes, 1997). It was of interest to determine if the uptake of these known iron sources for *Bordetella* requires TonB function and if TonB function is required by these organisms during iron-limited conditions. It is likely that *Bordetella* species require the ability to multiply during iron scarcity in order to establish infection.

METHODS

Plasmids and strains. These are described in Table 1.

Media, growth conditions and antibiotics. Luria-Bertani (LB) medium was used for growth of *Escherichia coli* and nonhaemolytic *B. bronchiseptica* strains B013N and 19385. Bordet Gengou agar with 5% sheep blood was used for *B. bronchiseptica* haemolytic strain F4178 and its derivatives. All *Bordetella* strains were assessed for the production of haemolysin by growth on Bordet Gengou medium. Stainer-Scholte minimal medium (von Koenig *et al.*, 1988) lacking added iron supplement (SS) was used for growth of *B. pertussis*. LB agar

containing 100 µM DP (2,2'-dipyridyl) and SS containing 45 µg DP ml⁻¹ were used to test for growth under low iron availability in *B. bronchiseptica* and *B. pertussis* respectively. Iron-deficient medium for iron complex utilization bioassays was SS agar containing 45 µg EDDA [ethylenediamine-di(o-hydroxyphenylacetic acid)] ml⁻¹ (SS-EDDA). For iron source utilization assays, SS-EDDA was overlaid with 3 ml soft SS agar containing 10⁶ cells ml⁻¹. Disks containing 15 µl of different concentrations of iron sources were laid upon the surface, and growth haloes for *B. bronchiseptica* and *B. pertussis* strains were assessed after incubation at 37 °C for 16 h and 48 h respectively. Albomycin sensitivity was assessed in the same manner for both species, measuring zones of inhibition around albomycin-impregnated disks on SS agar overlays. LB agar and LB broth containing 60–250 µg EDDA ml⁻¹ were used for enterobactin disk bioassays and growth experiments with *E. coli tonB* and *fepA* mutants. LB agar plates containing 200 µM DP were also used for disk bioassays with *E. coli* mutants.

Tetracycline was used at 15 µg ml⁻¹ and ampicillin at 200 µg ml⁻¹. Gentamicin was used at 15 µg ml⁻¹ for *Bordetella*.

Iron sources. Enterobactin extracts were prepared as previously described (Porra *et al.*, 1972; Beall & Sanden, 1995b) and bioassay stocks were 50 µM. The source of alcaligin used for this work was SS broth culture supernatant obtained from the *B. bronchiseptica fur* mutant B013NMn⁺4 (Brickman & Armstrong, 1995). The following were obtained from Sigma and the indicated stock concentrations were used in bioassays: haemin (100 µM), ferrichrome (from *Ustilago sphaerogena*, 50 µM), desferrioxamine B [desferoxamine mesylate (desferal), 50 µM] and human haemoglobin (500 µg ml⁻¹).

Preparation of DNA. Total genomic DNA was extracted from *Bordetella avium*, *B. bronchiseptica*, *B. parapertussis* and *B. pertussis* as previously described (Beall & Sanden, 1995a). Plasmids were prepared with Qiagen mini-prep kits.

Southern analysis. This was performed as previously described (Beall & Sanden, 1995a). The degenerate oligonucleotides [CCNGAR]3 and [AARCCN]3, used to detect *tonB*, were 3'-end-labelled with digoxigenin (NCID/CDC Biotechnology Core Facility Branch), and were used as probes for DNA hybridization at 42 °C as described in the Genius kit (Boehringer Mannheim). Gene-specific probes were derived from pMLN1 containing the 6 kb chromosomal *Pst*I fragment from *B. bronchiseptica*. The fragments were randomly labelled with digoxigenin (Genius kit) and used as probes for high-stringency DNA hybridization at 68 °C, as described by the manufacturer.

DNA sequencing. Plasmid pMLN1 and appropriate plasmid subclones were sequenced with ABI dye-deoxy terminator kits on an ABI 377 sequencer as described by the manufacturer (Applied Biosystems) using oligonucleotides annealing to the chromosomal inserts or the pUC19 multiple cloning site. The GCG Sequence Analysis Software Package (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, WI, USA) was used to analyse the DNA sequence.

Transformation and conjugation. Plasmids were transformed into *E. coli* by standard methods. Chromosomal integration of plasmids into *B. pertussis* and *B. bronchiseptica* was facilitated by conjugation with *E. coli* donor strain SM10 as previously described (Stibitz, 1994; Beall & Hoenes, 1997), except that counter-selection employed colicins B and Ia (Brickman & Armstrong, 1996b).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or characteristics*	Source or reference
<i>E. coli</i>		
SM10	RP4-2 Tc::Mu conjugation donor strain	Stibitz (1994)
DH10B	<i>mcrA</i> Δ <i>mcrBC</i> Δ <i>hdsR</i> Δ <i>hdsM</i> <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>lacZ</i> Δ M15	Gibco-BRL
H306	<i>tonB</i>	K. Hantke, Tübingen University
MT912	<i>thi</i> <i>trp</i> <i>purE</i> <i>proC</i> <i>leuB</i> <i>lacY</i> <i>mtl</i> <i>xyl</i> <i>rpsL</i> <i>azi</i> <i>fhuA</i> <i>tsx</i> <i>supA</i> <i>fepA</i> Kan ^R	M. McIntosh, E. Carolina University School of Medicine
H5058	<i>tsx</i> <i>aroB</i> <i>cir</i> <i>fiu</i> <i>fepA</i>	K. Hantke
<i>B. bronchiseptica</i>		
19385	ATCC 19385 NaI ^R (dog isolate), nonhaemolytic	Beall & Hoenes (1997)
B013N	Pig isolate, nonhaemolytic	Brickman & Armstrong (1995)
B013NMn ^R 4	B013N <i>fur</i>	Brickman & Armstrong (1995)
F4178	Human throat isolate, β -haemolytic	CDC collection
<i>B. pertussis</i>		
82	Wild-type clinical isolate, β -haemolytic	Beall & Sanden (1995b)
UT25D	Derivative of UT25, nonhaemolytic	Brown & Parker (1987)
<i>B. parapertussis</i>		
A168	Wild-type clinical isolate	Beall & Sanden (1995b)
<i>B. avium</i>		
BA10	Wild-type	Beall & Sanden (1995b)
Plasmids		
pSS2141	Broad-host-range integrational vector, Gm ^R Ap ^R	Stibitz (1994)
pRK415	Broad-host-range replicative vector, Tet ^R	Keen <i>et al.</i> (1988)
pRKTon	pRK415 derivative containing <i>B. bronchiseptica</i> <i>tonB-exbB-exbD</i> operon	This work
pSS2t, pSS23	pSS2141 containing <i>tonB</i> gene fragment for integrational inactivation of <i>tonB</i> _{Bb}	This work
pSS2t1	pSS2141 containing 5' end of <i>tonB</i> _{Bb} gene	This work
pKp1	PUC19 derivative containing <i>bfeA</i> and promoter	Beall & Sanden (1995b)

* Ap^R, Gm^R, Kan^R, NaI^R, Tet^R, denote resistance to ampicillin, gentamicin, kanamycin, nalidixic acid and tetracycline respectively.

RESULTS

Sequence of the *B. bronchiseptica* *tonB-exbB-exbD* genes

In order to clone the *tonB* gene from *B. bronchiseptica*, we designed two degenerate oligonucleotide probes, [CCNGAR]3 and [AARCCN]3, corresponding to prominent (Glu-Pro)_n and (Lys-Pro)_n repeats characteristic of many TonB proteins. This strategy relied upon identifying identically sized genomic restriction fragments that hybridized in independent Southern blots with each of the two degenerate primers. In this way, a 6 kb *Pst*I fragment was identified (Fig. 1), and subsequently cloned into pUC19 to generate pMLN1.

The complete nucleotide sequence of the 2.4 kb *Sma*I DNA fragment situated within the 6 kb *Pst*I fragment was determined. An examination of the sequence revealed three open reading frames directed in the same orientation. Because of obvious homology with characterized proteins, these open reading frames were sequentially designated *tonB*_{Bb}, *exbB*_{Bb} and *exbD*_{Bb}. As in *Neisseria* species, *Haemophilus influenzae*, *Pseudomonas putida* and *Xanthomonas campestris*, these genes appear to be organized in an operon in *B. bronchiseptica*

(Bitter *et al.*, 1993; Jarosik *et al.*, 1994; Stojiljkovic & Srinivasan, 1997; Wiggerich *et al.*, 1997). The *exbB*_{Bb} and *exbD*_{Bb} genes are predicted to have efficient ribosome-binding sites compared to the ribosome-binding site for *tonB*_{Bb}. This feature is likely to be essential in production of sufficient levels of these proteins for efficient TonB activation.

The 268-codon *tonB*_{Bb} showed strong homology to various Gram-negative TonB proteins, with 30–39% identity between overlaps of about 200–270 residues. These included the TonB proteins from *E. coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Serratia marcescens*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *P. putida*, *X. campestris*, *H. influenzae* and *Neisseria meningitidis* (GenBank accession numbers K00431, X56434, X68478, A36928, X60996, Q05741, U23764, S28444, Z95386, U04996 and U77738 respectively). A feature of TonB_{Bb} shared with other TonB proteins includes a short hydrophobic N-terminal putative transmembrane anchor (residues 20–40) with the remainder constituting a mainly hydrophilic putative periplasmic domain. Like other TonB proteins, the *B. bronchiseptica* TonB is proline-rich (21%) and exhibits Lys-Pro and Glu-Pro

Fig. 1. Southern blot of *B. bronchiseptica* DNA digests using degenerate oligonucleotides [CCNGAR]3 (a) and [AARCCN]3 (b) as digoxigenin-labelled probes. DNA was digested with *Accl* (lane 1), *AatII* (lane 2), *BamHI* (lane 3), *EcoRI* (lane 4), *PstI* (lane 5), *SmaI* (lane 6) and *XhoI* (lane 7). The 6 kb *PstI* fragment containing the *tonB-exbB-exbD* operon is indicated by an x.

Fig. 2. Diagram of the *tonB-exbB-exbD* region of *B. bronchiseptica*. A putative promoter and direction of transcription is indicated by an arrow. Two potential terminators are labelled 't'. *hupA'* and *orf'* represent two truncated open reading frames described in the text. Black rectangles represent gene-specific probes used for the Southern analysis shown in Fig. 3. pSSst1 and pSSst2 indicate Campbell-type insertions of the indicated plasmids into *Bordetella* strains with the iron-utilization phenotype (TonB⁺ or TonB⁻) shown. The entire *EcoRV-BamHI* fragment present in the replicative plasmid pRKton used for complementation studies is represented. Restriction enzymes: A, *AatII*; B, *BamHI*; C, *Clal*; E, *EcoRI*; N, *NcoI*; S, *Sall*; Sm, *SmaI*; X, *XhoI*.

repeats (7 and 12 repeats respectively) typical of previously described TonB proteins.

Located 1 bp downstream of the *tonB* stop codon was

the 314-codon *exbB_{Bb}* gene, which has deduced homology to various ExbB proteins (about 29–41 % sequence identity over 85–286 residues). The closest matching ExbB homologue to ExbB_{Bb} is from *X. campestris*, with 41 % sequence identity (GenBank accession number Z95386).

The 155-codon *exbD_{Bb}* gene overlapped by 1 base with the *exbB_{Bb}* stop codon. The deduced ExbD_{Bb} protein sequence shows the most similarity to the *N. meningitidis* and *X. campestris* homologues, with 36–40 % identity (GenBank accession numbers U77738 and Z95386 respectively).

Directly upstream of *tonB_{Bb}* are potential –35 and –10 hexamers homologous to the consensus sequence of *E. coli* σ^{70} -directed promoters (Hawley & McClure, 1983). This putative promoter sequence is overlapped by a sequence similar to Fur-binding sites. The sequence from bases 86 to 105 (see accession AF087669) is identical to the consensus Fur-binding site in 12 of 19 positions (Calderwood & Mekalanos, 1987), and was the only 'iron box' found with a homology search of the entire 2446 bp. About 160 bp upstream of *tonB* are two G + C-rich inverted repeats between bases 10–39, either of which could possibly function in transcription termination. Partial sequence analysis of the open reading frame immediately upstream of the sequence shown in accession AF087669 revealed a gene highly homologous to genes encoding bacterial histone-like proteins, including *S. typhimurium* *hupA* (Higgins & Hillyard, 1988). Eighty bases downstream of *exbD* and down-

Fig. 3. Southern analysis of *B. avium* (lane 1), *B. bronchiseptica* (lane 2), *B. parapertussis* (lane 3) and *B. pertussis* (lane 4) digested with *Aat*II and probed with the gene-specific probes *tonB* (a), *exbB* (b) and *exbD* (c).

stream of a potential transcriptional terminator was an open reading frame with high homology to various 'two-component system' DNA-binding regulatory proteins (Fig. 2).

Conservation of the *tonB*–*exbB*–*exbD* operon in *Bordetella* species

The *tonB*_{Bb}, *exbB*_{Bb}, *exbD*_{Bb} genes and flanking regions appear to be highly conserved between *B. bronchiseptica*, *B. pertussis* and *B. parapertussis*, since DNA from each of these three species had apparently identically sized *Aat*II restriction fragments that hybridized strongly to the three gene fragment probes (Fig. 3a–c, lanes 2–4). In addition, *B. bronchiseptica* and *B. pertussis* shared identically sized *Eco*RI and *Xho*I restriction fragments that hybridized to each of the gene fragment probes (data not shown). Since *Xho*I, *Eco*RI and *Aat*II have single sites within the *tonB*_{Bb}, *exbB*_{Bb} and *exbD*_{Bb} genes respectively (Fig. 3), these results indicate that these three genes and their flanking regions are highly conserved between the two species. These results also show that the putative *B. avium* homologues of these genes are not detectable by high-stringency Southern analysis (Fig. 3a–c, lane 1).

Construction of *B. bronchiseptica* and *B. pertussis* *tonB* mutants

Two chromosomal fragments were used for plasmid integrational analysis of the *tonB* gene in *B. bronchiseptica* and in *B. pertussis* (Fig. 2). Plasmid pSSt1 was constructed by cloning a 294 bp PCR fragment consisting of bases 209–502 including the first 60 codons of *tonB*_{Bb} and 4 bp of upstream sequence, into the *Eco*RI site of pSS2141 (Stibitz, 1994) immediately downstream of the gentamicin-resistance gene such that the *tonB* fragment was in the same orientation as the gentamicin-resistance gene. Plasmids pSSt2 and pSSt3 consisted of the 506 bp internal *tonB*_{Bb} *Xho*I–*Sal*I fragment cloned into the *Sal*I site of pSS2141 in either orientation (bases

345–851). These plasmids were subsequently integrated into the chromosome of *B. bronchiseptica* strains 19385, B013N and F4178 by conjugation with the *E. coli* donor strain SM10 followed by selection for colicin and gentamicin resistance. Since pSSt2 and pSSt3 contained an internal fragment lacking both the 5' and 3' ends of the *tonB*_{Bb} structural gene, simple homologous insertion of the plasmid resulting from a single crossover event within the *tonB* gene resulted in a truncated *tonB*_{Bb} gene of 218 codons.

Attempts to insertionally inactivate *tonB* in the clinical *B. pertussis* strain 82 with plasmids pSSt2 and pSSt3 were unsuccessful. Transconjugants were obtained through matings of strain 82 with SM10(pSSt2) and SM10(pSSt3), but at a very low frequency. Chromosomal DNA was prepared from one transconjugant, digested with *Eco*RI, and then ligated. This was followed by transformation of *E. coli* DH10B to ampicillin resistance. Plasmid DNA from several transformants from this chromosome-walking procedure revealed that these plasmids did not contain restriction fragments of the sizes predicted for pSSt2 or pSSt3 derivatives that had integrated into the strain 82 *tonB* gene (data not shown). In contrast, transconjugants were readily obtained by conjugation of plasmids pSSt2 and pSSt3 into *B. pertussis* strain UT25D. This strain is a non-haemolytic derivative of UT25 (Brickman & Armstrong, 1995) that has been adapted to grow on LB agar. In contrast to strain 82 transconjugants, plasmid pSSt2 and pSSt3 integrations into UT25D and the two *B. bronchiseptica* wild-type strains were found to occur as predicted by a single homologous crossover event. This resulted in truncation of 50 C-terminal residues of TonB in the *B. bronchiseptica* strains and a similar if not identical truncation of TonB in *B. pertussis* strain UT25D.

Our stock *B. bronchiseptica* strains 19385 and B013N were non-haemolytic on Bordet Gengou agar, possibly due to laboratory passage resulting in phase variation or modulation (Weiss & Hewlett, 1986), although it has

Table 2. TonB-dependent phenotypes in wild-type and Campbell insertion derivatives of *B. bronchiseptica* and *B. pertussis*

Strain*	<i>tonB</i>	LB-DP†	SS-DP‡	Iron utilization§	Albomycin
19385	+	+	+	+	S
19340 (19385ΩpSSt1)	+	+	+	+	S
19341 (19385ΩpSSt2)	—	—	—	—	R
19341(pRKton)	+	+	+	+	S
19341(pRK415)	—	—	—	—	R
B013N	+	+	+	+	ND
BN600 (B013NΩpSSt2)	—	+	+	—	ND
F4178	+	+	+	+	S
F4179 (F4178ΩpSSt2)	—	—	—	—	R
UT25D	+	ND	+	+	S
BB100 (UT25DΩpSSt2)	—	ND	—	—	R
BB100(pRKton)	+	ND	+	+	S
BB100(pRK415)	—	ND	—	—	R
82	+	ND	—	+	S

ND, Not determined.

* The symbol Ω indicates that the strain contains a single homologously inserted copy of the indicated plasmid.

† Growth on LB agar containing 100 µg dipyrindyl ml⁻¹.

‡ Growth on SS agar containing 45 µg dipyrindyl ml⁻¹.

§ Growth haloes in iron utilization assays around disks containing alcaligin, enterobactin, ferrichrome, desferrioxamine B, haemin or haemoglobin.

|| Sensitivity (S) indicates zones of inhibition around albomycin disks containing 10 µl of a 12.5 µg ml⁻¹ albomycin solution. Resistance (R) indicates no zones of inhibition using 10 µl of 250 µg ml⁻¹ albomycin.

been observed that initial isolates of *B. bronchiseptica* from clinical specimens are often non-haemolytic. Since we were unable to insertionally inactivate *tonB* in the clinical, haemolytic *B. pertussis* isolate 82, it appeared possible that attempts to insertionally inactivate *tonB* in phase 1 haemolytic strains of *B. bronchiseptica* would be unsuccessful. However, insertional inactivation of *tonB* in the β-haemolytic *B. bronchiseptica* human isolate F4178 was readily accomplished.

Iron source utilization deficiency and albomycin sensitivity of *tonB*_{Bb} mutants

Integration of pSSt1, which contained the 5' end of the structural *tonB*_{Bb} gene, into the *B. bronchiseptica* chromosome resulted in placing the intact *tonB*_{Bb} gene immediately downstream of the pSS2141 gentamicin-resistance gene and in the same orientation. This also resulted in replacement of the *tonB* ribosome-binding site with a different ribosome binding region (from ACCTGGCTGATTC-ATG to TAATTAAGAATTC-ATG). Campbell-type insertion of pSSt1 had no effect on the ability of *B. bronchiseptica* strain 19340 to grow on LB agar containing 100 µM DP, nor did these transconjugants differ from the parental strain 19385 in utilization of various iron sources as measured by disk bioassays (Table 2). Therefore, the vector promoter sequence(s) and the different *tonB*_{Bb} ribosome-binding

site resulting from Campbell insertion of pSS2141 served to substitute for the normal *tonB*_{Bb} promoter and ribosome-binding site.

In contrast, Campbell insertions of pSSt2 or pSSt3 resulted in the inability of *B. bronchiseptica* and *B. pertussis* UT25D transconjugants to form growth haloes in disk-bioassays around alcaligin, enterobactin, ferrichrome, desferrioxamine B and haemin (Table 2). Further indication of an alcaligin-utilization defect in transconjugant strain 19341 was its inability to grow on LB-DP and SS-DP. It was interesting to note that pSSt2 and pSSt3 Campbell integration derivatives of the dog isolate B013N were unable to utilize the various iron sources used in bioassays, but were unimpaired in their ability to grow on LB-DP and SS-DP agar. It was also interesting that the clinical *B. pertussis* isolate strain 82 was unable to form colonies on SS-DP agar, yet was able to utilize all of the various iron sources used in bioassays (Table 2). These observations indicate differences among strains of these species affecting growth in iron-limiting media.

Another phenotype demonstrating *tonB* inactivation in *B. bronchiseptica* and *B. pertussis* *tonB* mutant strains was their marked resistance to the antibiotic albomycin (Table 2), which is structurally similar to ferrichrome. These strains showed no zones of growth inhibition around albomycin disks containing 10 µl of a 250 µg

ml⁻¹ albomycin solution, while the wild-type parental strains showed wide zones of inhibition around disks containing 10 µl of 25 µg ml⁻¹ albomycin (data not shown).

Complementation of *B. bronchiseptica* and *B. pertussis* tonB mutants

A 3.6 kb *EcoRV*–*Bam*HI fragment from pMLN1 containing the intact *tonB*_{Bb}–*exbB*_{Bb}–*exbD*_{Bb} operon (Fig. 2) was subcloned into the broad-host-range vector pRK415 (Keen *et al.*, 1988), resulting in plasmid pRKton. Introduction of pRKton into the *tonB* mutant strains *B. bronchiseptica* 19341 and *B. pertussis* BB100 completely restored wild-type iron-utilization and albomycin-sensitivity phenotypes (Table 2), demonstrating that the mutant phenotypes were not conferred by polarity effects on genes downstream of *exbD*.

tonB_{Bb} on pRKton cannot complement an *E. coli* tonB mutant

Plasmid pRKton could not complement the enterobactin and vitamin B12 utilization defects exhibited by the *E. coli* *tonB* mutant strain H306 (data not shown). The TonB-dependent receptors for colicin B, colicin Ia and albomycin were also still not functional in this mutant since H306(pRKton) was still totally resistant to each of these molecules (data not shown).

tonB_{Bb} and bfeA inefficiently reconstitute enterobactin utilization in an *E. coli* fepA mutant

Attempts were made to reconstitute enterobactin utilization in two different *E. coli* *fepA* mutants [H5058 (*fepA*, *aroB*) and MT912 (*fepA*)] and in H306(*tonB*) harbouring the compatible plasmids pKp1 and pRKton. With H5058, H306 and their plasmid-containing derivatives, no differences were seen in disk bioassays using added enterobactin, nor were differences seen in growth assays in iron-limiting media (data not shown). However, the *fepA* mutant MT912(pKp1, pRKton) formed larger colonies than MT912(pKp1, pRK415) on LB agar containing 60 µg EDDA ml⁻¹ and this effect was measurable in LB broth containing EDDA (data not shown). These effects in liquid medium were at least partially due to a higher colony-forming efficiency of MT912(pKp1, pRKton) relative to MT912(pKp1, pRK415) in media containing 100–250 µg EDDA ml⁻¹ (10⁻⁴ vs 0 colony-forming efficiency). We also found small-diameter growth haloes around enterobactin disks with MT912(pKp1, pRKton) on L agar containing 250 µg EDDA ml⁻¹ within 24 h, whereas MT912(pKp1, pRK415) only displayed a very faint growth halo after 72 h. This latter effect is likely to be due to Cir- and Fiu-mediated utilization of enterobactin degradation products (Hantke, 1990).

DISCUSSION

Although the *B. bronchiseptica* TonB, ExbB and ExbD proteins share only 34%, 29% and 32% sequence identity respectively with corresponding overlaps with

their respective *E. coli* homologues, each of these homologue pairs have strikingly similar hydrophobicity plots, which probably reflects conserved cellular localization and function. The majority of identified TonB proteins contain Pro-Glu and Pro-Lys repeat domains in close proximity, upon which we relied for our strategy in cloning *tonB*_{Bb}. The region encompassing these domains is thought to allow TonB to physically extend through the periplasm, allowing contact with outer-membrane receptors (Evans *et al.*, 1986; Hannavy *et al.*, 1990; Larsen *et al.*, 1993). The histidine at residue 20 and neighbouring residues conserved among other TonB residues are believed to be important in the *E. coli* TonB function (Karlsson *et al.*, 1993; Jaskula *et al.*, 1994). There is a similar region centring at the histidine residue in TonB_{Bb} (residue 30) that is situated at roughly the same position in the putative membrane-associated N-terminus at nearly the same distance from a conserved arginine at residue 19. The PXYP motif (residues 160–163 in the *E. coli* TonB; 190–193 in TonB_{Bb}) thought to interact with outer-membrane TonB boxes (Heller *et al.*, 1988; Gudmundsdottir *et al.*, 1989; Kadner, 1990; Traub *et al.*, 1993) is conserved among most of the TonB proteins, including TonB_{Bb}, which is consistent with the similarity of *Bordetella* siderophore-receptor-like protein TonB boxes to those of *E. coli* TonB-dependent receptors. Two proposed amphiphilic β-strands (Anton & Heller, 1991) (encompassed between residues 173–196 for the *E. coli* TonB and 197–215 for TonB_{Bb}) with highly conserved glycine residues (positions 174 and 186 for the *E. coli* TonB, 203 and 215 for TonB_{Bb}) are conserved among all of the known TonB proteins.

As with the *P. putida* ExbB protein, ExbB_{Bb} has a non-conserved N-terminal extension relative to other ExbB proteins. However, both of these ExbB proteins share highly conserved α-helical transmembrane domains with the other known ExbB proteins. It is within these domains that ExbB is thought to interact with TonB and ExbD (Kampfenkel & Braun, 1992; Koebnik, 1993; Larsen *et al.*, 1994; Traub *et al.*, 1993). These include the N-terminal region VX₃VX₃LX₃SX₃ motif (residues 84–102 of ExbB_{Bb}), a domain encompassed by residues 202–225 of ExbB_{Bb} with four conserved glycine residues, and an alanine-rich α-helical region between residues 240–270 with conserved asparagine and arginine residues (residues 265 and 270).

The most conserved regions between ExbD_{Bb} and other ExbD proteins are the putative N-terminal transmembrane region (approx. residues 17–40) and the C-terminal periplasmic region thought to interact with TonB and ExbB. The aspartate residue (residue 25 and 26 of the *E. coli* and *B. bronchiseptica* ExbD respectively), shown to be important in ExbD function (Braun *et al.*, 1996), is conserved among known ExbD proteins; and the *E. coli* ExbD leucine 132, also shown to be functionally important (Braun *et al.*, 1996), is conservatively substituted by phenylalanine in *B. bronchiseptica* (residue 133), *X. campestris* and *N. meningitidis*. It is unknown why we were unable to insertionally

inactivate *tonB* in a phase 1 isolate of *B. pertussis*. We have subsequently attempted *tonB* inactivation in an independent phase 1 isolate with no success (data not shown). It is likely that this result is due to an effect of *tonB* inactivation on processes other than iron uptake. For example, in *Pseudomonas aeruginosa*, *tonB* inactivation results in hypersensitivity to a wide variety of antibiotics in a manner that is independent of iron concentration in the growth medium (Zhao *et al.*, 1998), although we did not note problems in selecting gentamicin resistance for the insertional inactivation of *tonB* in the phase 1 *B. bronchiseptica* strain F4178.

The *P. aeruginosa* TonB protein does not appear significantly more related to the *E. coli* TonB than TonB_{Bb}, yet the *P. aeruginosa* TonB is known to function in *E. coli tonB* mutants, facilitating the utilization of several different receptors (Poole *et al.*, 1996). TonB proteins of *Neisseria* species show the same overall conservation of key sequence features with the *E. coli* TonB; however, the *N. meningitidis* TonB apparently cannot complement *E. coli* in the utilization of its TonB-dependent receptors (Stojiljkovic & Srinivasan, 1997). Reconstitution of the *N. meningitidis* haemoglobin-utilization system in *E. coli* was successful, employing expression of the *N. meningitidis* haemoglobin-receptor gene and *tonB-exbB-exbD* operon in *E. coli* (Stojiljkovic & Srinivasan, 1997). In analogous experiments, we attempted to reconstitute enterobactin utilization in an *E. coli tonB* mutant and in *E. coli fepA* mutants. We introduced the *Bordetella tonB-exbB-exbD* operon and *fepA* homologue, *bfeA*, into these mutants on compatible plasmids; however, we were only able to demonstrate apparently very inefficient reconstitution in one *fepA* mutant. Among possible explanations for this result are that the level of *bfeA* and/or *tonB-exbB-exbD* expression is inadequate in these strains, or that these proteins are unstable in *E. coli*. In previous experiments where *bfeA* was expressed in *E. coli* from the powerful T7 promoter and radioactively labelled, the level of labelled BfeA was very low, indicating either poor translation or perhaps degradation of BfeA (Beall & Sanden, 1995b). Another possible explanation is the formation of inactive hybrid TonB-ExbB-ExbD complexes.

The results shown in this work further suggest the presence of several different TonB-dependent receptors in *B. bronchiseptica* and *B. pertussis*, since *tonB* mutants of these species were unable to utilize various iron complexes (Table 2). These receptors include the ferric enterobactin receptor (BfeA), and unidentified receptors for ferric complexes of alcaligin, ferrichrome and desferrioxamine B. Additionally, there must be at least one TonB-dependent receptor for haem sources, since utilization of haemin and haemoglobin was TonB dependent in *B. bronchiseptica* and *B. pertussis* (Table 2). Together with the iron-regulated BfrA, BfrB and BfrC proteins that have unknown ligand specificity, this brings the minimal number of predicted TonB-dependent outer-membrane receptors in this genus to eight. Since the only known siderophore produced by

Bordetella species is alcaligin (Moore *et al.*, 1995; Brickman & Armstrong, 1996a), the biological significance of the complex array of exogenous iron complex utilization systems present in the genus *Bordetella* is as yet unknown.

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