Bordetella pertussis TonB, a Bvg-Independent Virulence Determinant

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Received 23 August 1999/Returned for modification 21 October 1999/Accepted 20 December 1999

In gram-negative bacteria, high-affinity iron uptake requires the TonB/ExbB/ExbD envelope complex to release iron chelates from their specific outer membrane receptors into the periplasm. Based on sequence similarities, the Bordetella pertussis tonB exbB exbD locus was identified on a cloned DNA fragment. The tight organization of the three genes suggests that they are cotranscribed. A putative Fur-binding sequence located upstream from tonB was detected in a Fur titration assay, indicating that the tonB exbB exbD operon may be Fur-repressed in high-iron growth conditions. Putative structural genes of the β-subunit of the histone-like protein HI and of a new two-component regulatory system were identified upstream from tonB and downstream from exbD, respectively. A B. pertussis ΔtonB exbB::Km" mutant was constructed by allelic exchange and characterized. The mutant was impaired for growth in low-iron medium in vitro and could not use ferrichrome, desferal, or hemin as iron sources. Levels of production of the major bacterial toxins and adhesins were similar in the TonB"/TonB+ pair. The ΔtonB exbB mutant was still responsive to chemical modulators of virulence, thus, the BvgA/BvgS two-component system is not TonB dependent. Nevertheless, in vivo in the mouse respiratory infection model, the colonization ability of the mutant was reduced compared to the parental strain.

Most bacteria require an iron concentration of 10^-6 to 10^-8 M for growth. In the host, iron is not readily available to microorganisms since Fe(III) is bound to transferrin (TF) in the serum and to lactoferrin (LF) in other secretions. The concentration of free iron in body fluids is estimated to be less than 10^-18 M; thus, the ability of a pathogen to scavenge iron may represent an important virulence trait (65). Some bacteria, e.g., Neisseria spp. and Haemophilus influenzae, produce cell surface receptors for TF, LF, heme, or heme-containing proteins (10, 24, 37, 53). Others, e.g., Escherichia coli and Pseudomonas spp., secrete low-molecular-weight iron chelators termed siderophores which are able to remove Fe(III) from TF or LF (44). Iron-loaded siderophores can then bind to high-affinity receptors on the bacterial cell surface, and be internalized. In gram-negative bacteria the TonB/ExbB/ExbD complex, referred to as the Ton system, interacts with the outer membrane receptors involved in iron uptake and transduces the energy required for the transfer of Fe(III) from TF and LF or that of heme or ferrisiderophores into the periplasm. TonB is anchored in the inner membrane, where it is stabilized by the ExbB and ExbD proteins (for a review, see reference 43). Vitamin B12, group B cobalins, and certain phages are also delivered into the cell via specific receptors and the Ton system in E. coli (13, 30, 50). Through a cycle of conformational changes TonB couples the cytoplasmic membrane proton motile force to active transport across the outer membrane (35).

We were interested in deciphering the iron uptake systems and the potential influence of the iron regulatory network in virulence in bordetellae. Bordetella pertussis, the etiologic agent of whooping cough, Bordetella parapertussis, which infects humans and sheep, and Bordetella bronchiseptica, the causative agent of swine atrophic rhinitis and kennel cough, synthesize alcaligin, a hydroxamate-type siderophore. Bordetella avium, a poultry pathogen, does not seem to produce siderophore (17, 51). We and others independently identified and characterized alcR, the gene encoding an AraC-type activator of the alcaligin biosynthesis operon in B. pertussis, B. parapertussis, and B. bronchiseptica (6, 51). The expression of the recently identified alcaligin receptor gene is also AlcR regulated in B. bronchiseptica (6, 14). Surprisingly, the virulence of a B. pertussis alcR null mutant is not impaired in the mouse respiratory infection model (51). This observation suggests that B. pertussis possesses alcaligin-independent iron uptake systems which may contribute to efficient colonization of the host. An LF-binding protein has been detected in membrane fractions of bordetellae, but its role in iron uptake has not been established yet (40). In addition, several exogenous siderophore receptors have been identified in B. pertussis. These include BfeA, which binds enterobactin, and BfrB and BfrC, the receptors for unknown siderophores (3, 5). A B. bronchiseptica-specific receptor, BfrA, has also been characterized, but its ligand remains unidentified (4). B. pertussis is also able to use hemin as a sole iron source, suggesting that it produces an outer membrane heme receptor (4). Heme uptake is Ton dependent in several pathogens (29, 38, 60, 62), although in Neisseria gonorrhoeae and Haemophilus ducreyi heme uptake does not require the Ton system (8, 19). In order to evaluate the role of the Ton system in B. pertussis iron uptake and virulence, we first identified and characterized the tonB exbB exbD locus in this species. A B. pertussis ΔtonB exbB mutant was constructed and compared to the parental strain. The mutant presented reduced growth in iron-depleted medium and was deficient in exogenous siderophores, hemin and albomycin uptake. The mutant was also impaired in its ability to colonize the respiratory tract of infected mice, although the expression and in vitro regulation of Bvg-dependent virulence factors proved to be unaffected. Thus, our data suggest that TonB-dependent transport systems are important, yet Bvg-independent virulence traits in B. pertussis.
TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant features*</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>E. coli RRK5048</td>
<td>metE70 tonB</td>
<td>26</td>
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<td></td>
<td>arsR flaF3 Δ placMu; Km'</td>
<td>58</td>
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<tr>
<td>XLI-Blue</td>
<td>High efficiency transformation; Te'</td>
<td>57</td>
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<td>SM10</td>
<td>Mobilizing strain; Km'</td>
<td>Novagen</td>
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<tr>
<td>BL21(DE3)/pLYS S</td>
<td>High-stringency expression host; Cm'</td>
<td>Institut Pasteur, Paris, France</td>
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<tr>
<td>B. avium 103004</td>
<td>Sm' but not rpsL</td>
<td>51</td>
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<tr>
<td>B. parapertussis PEP</td>
<td>Sm'</td>
<td>45</td>
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<td>pSU19 containing E. coli tonB, exbB, and exbD; Cm'</td>
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* Ap', Cm', Gm', Km', Nal', Sm', and Te', resistance to ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, streptomycin and tetracyclin, respectively.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this work are listed in Table 1. E. coli strains were grown at 37°C in Luria-Bertani (LB) medium (42) or on solid media obtained by addition of 1.5% (wt/vol) Bacto-Agar. In the Fur titration assay (58), the Lac phenotype of E. coli strains were usually grown in modified Stainer-Scholte (SS) medium containing 0.15 g of glutathione, and 10 mg of FeSO₄ 7H₂O. Low-iron medium was SS supplemented at 37°C on Bordet-Gengou (BG) agar base plates supplemented with 1% glycerol and 15% sheep blood. Liquid cultures were usually grown in modified Stainer-Scholte (SS) medium containing (per liter): 11.84 g of Na₂-gluatamate · H₂O, 0.24 g of L-proline, 2.5 g of NaCl, 0.5 g of KH₂PO₄, 0.2 g of KCl, 0.1 g of MgSO₄ · 7H₂O, 20 mg of CaCl₂ · 2H₂O, 1.5 g of Tris, 10 mg of Casamino Acids, 1 g of dimethyl β-cyclodextrin (a gift from D. R. Bartlett, University of California, Berkeley, Calif.), 40 mg of L-tryptophan, 4 mg of nicotinic acid, 0.4 g of ascorbic acid, 0.15 g of glutathione, and 10 mg of FeSO₄ · 7H₂O. Low-iron medium was SS without addition of FeSO₄ · 7H₂O. DNA ligases were usually grown on SS-Fe. Some growth tests were performed in Casamino Acid-free SS medium or in SS supplemented with only 0.1% Casamino Acids. Modulation conditions were obtained by the addition of 50 mM MgSO₄ or 5 mM nicotinic acid to SS. When necessary, antibiotics were included in the growth media at the following final concentrations: ampicillin (Ap), 150 mg/ml; chloramphenicol (Cm), 30 mg/ml; gentamicin (Gm), 10 mg/ml; kanamycin (Km), 30 µg/ml; nalidixic acid (Nal), 30 µg/ml; streptomycin (Sm), 100 µg/ml.

DNA techniques. Plasmid DNA was routinely isolated by the alkaline lysis method (55) or purified by using the Nucleobond AX kit (Macherey-Nagel, Hoerdt, France) for sequencing purposes. Restriction endonucleases and T4 DNA ligase were obtained from Roche (Meylan, France) and used according to standard procedures (55). DNA fragments were sequenced using an ABI PRISM Dye Terminator Cycle Sequencing kit and an ABI PRISM 377 sequencer (PE Applied Biosystems, Warrington, United Kingdom) and a combination of universal, reverse, and custom-synthesized primers. PCRs were carried out with Vent DNA polymerase (New England Biolabs, Inc., Beverly, Mass.).

Computer analysis of sequences. The nucleotide and protein sequences were analyzed by using the DNA Strider 1.2 software (Service de Biochimie et de Généétique Moléculaire du CEA, Saclay, France). Sequence similarities were identified with the help of the BLASTN and BLASTP programs (2). Sequence alignments were performed with the Multalin 5.3.3 software (18). Oligonucleotides were designed using the Oligo 5.0 software (NBI, Plymouth, Minn.).

Construction of pCG475 and pEP491 and cloning of the B. bronchiseptica tonB upstream region. Plasmid pCG475 was isolated from a B. pertussis PEP genomic DNA library we had previously constructed in pUC18. The BamHI fragment bearing B. bronchiseptica tonB was isolated from pCG475 and cloned into EcoRI fragment of pEP487; Cm' This study.

Construction of the B. bronchiseptica tonB upstream region. Plasmid pCG475 was isolated from a B. pertussis partial genomic DNA library we had previously constructed in pUC18. The BamHI fragment was isolated from pCG475 and inserted into the unique BclI site in pCG475. The resulting plasmid, pPE487, was digested with EcoRI and the 5.3-kb fragment bearing tonB exbB; exbD basR was cloned into the Bordetella suicide vector pQ200mp18rpsL (Gm') to obtain pEP491 (Fig. 1B). E. coli SM10 was transformed with pEP491 and used as a donor in conjugation with B. bronchiseptica BB1015. Genomic DNA of a Gm' Km' BB1015 mutant bearing pEP491 inserted into the tonB locus was digested with NsiI, which does not cut pEP491, and ligated. The ligation mixture was used to transform E. coli XL1-Blue to gentamicin resistance. A recombinant plasmid resulting from the intramolecular ligation of a chromosomal NsiI fragment containing pEP491 was isolated. Restriction mapping of this plasmid permitted identification of the B. bronchiseptica tonB 5' region. A 2.4-kb EcoRI DNA fragment localized immediately upstream from the tonB locus was subcloned into pBSCK' to yield pEP532 (Fig. 1C).
molten SS-Fe plus 0.1% Casamino Acids plus 10 μM EDDHA plus 0.8% agarose and poured into petri dishes. Agarose was used in plates because B. pertussis did not grow well on agar plates. Wells (4 mm in diameter) were punched in plates with a sterile plastic pipette and filled with 20 μl of 15 μM solutions of FeSO₄, FeCl₃, ferrichrome (Sigma Aldrich, St. Quentin Fallavier, France), desferal (a gift from Ciba-Geigy, Rueil Malmaison, France), or hemin (Sigma Aldrich) in SS-Fe or with SS-Fe alone as a control. Diameter of growth zones around wells were measured after 24 h of incubation at 37°C. To test albomycin sensitivity, 20 μl of molten SS-Fe plus 0.1% Casamino Acids plus 0.8% agarose were seeded with 200 μl of cell suspension and poured into petri dishes. Filter paper disks impregnated with 10 μl of albomycin (50 μg/ml in SS-Fe; a gift from K. Hanke and H-P. Fiedler) or SS-Fe were applied to the surface. Growth inhibition was checked after 24 h of incubation at 37°C.

**Mouse respiratory infection model.** After 24 h of growth on BG plates, BPSM or BPEP98 cells were resuspended in saline. Mice were intranasally infected with 50 μl of suspension containing 2 × 10⁷ bacteria. Infected mice were sacrificed by cervical dislocation 2 h after infection and at 5, 8, 12, and 16 and 22 days thereafter (two to four mice per time point). The lungs were removed and homogenized in saline with tissue grinders. Numeration of bacteria was performed by plating.B. To assess stability of the Δonb::KmR mutation, bacteria reisolated from the lungs of BPEP98-infected mice were tested for their resistance to Kmr and the absence of desferal, ferrichrome, or hemin utilization. All phenotypes had been retained.

**B. pertussis TonB production in E. coli.** B. pertussis TonB was overexpressed using the T7 RNA polymerase-promoter system (Novagen, Madison, Wis.). The tonB open reading frame (ORF) was amplified from pCG475 with primers NdeI-TonB (5'-ATATGATATGCCTAGCCCCCAATCTGGT-3') and TonB-EcoRI (5'-ATATGATATGCCTAGCCCCCAATCTGGT-3') by 35 PCR cycles of 1 min of denaturation at 95°C, 1 min of hybridization at 63°C, and 1 min of elongation at 72°C. The amplification product was digested with NdeI and EcoRI, and then cloned into pPET24a to obtain pPET24-TonB. pET24-TonB was transformed with either pET24a or pEP583 and grown in LB-Km-Cm at 37°C. At an OD₆₀₀ of 0.6, cells were induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) and grown for another 1 to 3 h before proteins were precipitated with trichloroacetic acid (TCA).

**Results**

**Cloning and sequence analysis of the B. pertussis tonB exbB exbD locus.** In the course of cloning in connection with another research project 6 years ago, we had isolated pCG475, a recombinant plasmid bearing a pCG475 3.1-kb EcoRI DNA fragment. Sequence analysis of the insert revealed the presence of four ORFs located on the same DNA strand (Fig. 1A). Similarity searches with the deduced amino acid sequences suggested that the first three ORFs encode homologues of TonB, ExbB, and ExbD and that the fourth one codes for a transcriptional activator of a bacterial two-component regulatory system. This latter ORF was called *tonB*. Similarity searches with the deduced amino acid sequences of four ORFs located on the same DNA strand suggested that the first three ORFs encode homologues of TonB, ExbB, and ExbD and that the fourth one codes for a transcriptional activator of a bacterial two-component regulatory system. This latter ORF was called *tonB*. Similarity searches with the deduced amino acid sequences of four ORFs located on the same DNA strand suggested that the first three ORFs encode homologues of TonB, ExbB, and ExbD and that the fourth one codes for a transcriptional activator of a bacterial two-component regulatory system. This latter ORF was called *tonB*. Similarity searches with the deduced amino acid sequences of four ORFs located on the same DNA strand suggested that the first three ORFs encode homologues of TonB, ExbB, and ExbD and that the fourth one codes for a transcriptional activator of a bacterial two-component regulatory system. This latter ORF was called *tonB*.
Fur in iron-rich growth conditions (22, 46, 48, 49). No obvious promoter sequence was identified in the short region upstream from tonB in pCG475. In addition, a pBCSK+ derivative containing the 3-kb EcoRI-XhoI tonB exbB exbD basR′ fragment from pCG475 conferred a Lac+ phenotype in the Fur titration assay, a genetic test for the presence of FBS using an E. coli indicator strain (58) (Fig. 1A). This suggested the absence of an FBS in the EcoRI-XhoI fragment and therefore that pCG475 does not contain the tonB promoter. A G+C-rich inverted sequence starting 27 bp downstream from the exbD stop codon (GCGCCGGCGCGGGCGGCGG) could constitute a termination signal for tonB exbB exbD transcription. Alternately, this sequence could play a role in basR expression, whose ORF starts 36 bp downstream from this hairpin structure with an ATG at position 2409. This ORF extends to position 3080, 60 bp upstream from the second EcoRI site (Fig. 1A).

The predicted sequences of B. pertussis TonB, ExbB, and ExbD were aligned with those of their respective closest homologues in the databases and with the corresponding E. coli sequences as a reference (Fig. 2). The B. pertussis TonB is a 268-amino-acid (aa) protein with a calculated molecular weight (MW) of 28,600. The highest degree of similarity was observed with Pseudomonas aeruginosa TonB (30% identity in a 275-aa overlap compared to 26% in a 240-aa overlap with E. coli TonB). Most TonB features are conserved: (i) a predicted N-terminal transmembrane segment (TM) containing a conserved His residue anchors TonB to the cytoplasmic membrane (Fig. 2A) and is involved in interactions of the E. coli TonB with ExbB (31, 35, 63); (ii) a Pro-rich domain, composed of 13 Glu-Pro and 9 Lys-Pro repeats in B. pertussis TonB, is proposed to span the periplasmic space in the case of E. coli TonB (36, 54). The remainder of TonB is a 325-aa protein with a calculated MW of 28,600. The highest degree of similarity was observed with Pseudomonas aeruginosa TonB (30% identity in a 141-aa overlap). ExbD proteins are predicted to has a PXYP motif and highly conserved Val and Gly residues are present in the C-terminal third of the protein (Fig. 2A). In E. coliTonB this region has been proposed to interact with outer membrane receptors (33, 63).

As a predicted 325-aa protein with a calculated MW of 33,800, B. pertussis ExbB is larger than most other ExbB proteins (220 to 240 aa). Its closest homologue is the Xanthomonas campestris ExbB (66), with 40% identity in a 253-aa overlap. In addition to the three TM domains typical of ExbB proteins, B. pertussis ExbB contains a putative fourth TM segment in its N-terminal extension (Fig. 2B). Such a characteristic is shared by the 329-aa Pseudomonas putida ExbB. Thus, in contrast to E. coli ExbB, the B. pertussis ExbB and P. putida ExbB N termini are predicted to be located in the cytoplasm. Apart from an Ala and Pro abundance, no striking sequence similarity could be detected between the N-terminal extensions of B. pertussis ExbB and P. putida ExbB. Most features of other ExbB proteins are conserved (59): (i) a VX3L/VX3LX3SX3W motif is present in the first common TM domain; (ii) the second conserved hydrophobic region is Gly-rich, while (iii) the last TM segment is Ala-rich and is followed by Asn and Arg residues present in most ExbB proteins (underlined in Fig. 2B).

B. pertussis ExbD is a predicted 155-aa protein with a calculated MW of 16,500. It is most similar to X. campestris ExbD1 (35% identity in a 140-aa overlap compared to 30% identity with E. coli ExbD in a 141-aa overlap). ExbD proteins are anchored in the inner membrane via a single N-terminal hydrophobic segment (Fig. 2C). An Asp residue required for E. coli ExbD activity is conserved in the B. pertussis ExbD TM domain (underlined in Fig. 2C). The remainder of E. coli ExbD extends into the periplasm where it interacts with TonB and ExbB. Leu132 in the C terminus of E. coli ExbD has been described as important for this activity (11). However, it is substituted by Phe in B. pertussis ExbD and X. campestris ExbD1 or by Ile in other ExbD proteins (21, 23, 28, 66).

While this manuscript was in preparation, Nicholson and Beall published an analysis of the B. bronchiseptica 19385 tonB exbBD locus. Their sequence data are very similar to ours except for 10 nucleotide changes and an 11-bp insertion in the B. pertussis BPSM exbBD intergenic region. We identified 20 nucleotide changes between the BPSM and 19385 tonB genes, but only two generate amino acid substitutions in the deduced protein sequences: V120A. However, due to a 33-bp in-frame deletion, the 19385 deduced protein sequences: V139A and A151T. Only 5 of the 30 differences observed between BPSM and 19385 exbB lead to amino acid substitutions: D24G, A27T, A47T, I103V, and 31 differences observed between BPSM and 19385 exbD.

FIG. 2. Sequence alignments of the deduced B. pertussis (Bp) TonB (A), ExbB (B), and ExbD (C) proteins with their closest homologues and the corresponding E. coli (Ec) proteins. Pa, P. aeruginosa; Xc, X. campestris. Predicted transmembrane segments are highlighted in gray. Conserved residues are in boldface, and those important for function in E. coli and conserved in the other two proteins are underlined.
generating residue substitutions E56Q and I65V and 14 silent ones.

**Presence of the tonB locus in other Bordetella genomes.** *B. parapertussis* and *B. bronchiseptica* are closely related to *B. pertussis*, while *B. avium* is phylogenetically more distant. The presence of the tonB exbB exbD genes in these species was tested in Southern blot experiments. Chromosomal DNA from *B. pertussis* BPSM, *B. bronchiseptica* BB1015, *B. parapertussis* PEP, and *B. avium* 103004 was digested with EcoRI and probed with the 3-kb EcoRI-XhoI DNA fragment of pCG47S containing the whole tonB exbB exbD locus. A single hybridization product was detected in each genome: a 3.1-kb DNA fragment in *B. pertussis* and *B. bronchiseptica* and a larger DNA fragment, of about 10 and 15 kb, in *B. parapertussis* and *B. avium*, respectively (data not shown). Thus, the tonB exbB exbD locus is present in these four species. Furthermore, both EcoRI sites flanking this region appear to be conserved in *B. pertussis* and *B. bronchiseptica*. Since, unlike the other three species, *B. avium* does not seem to synthesize the siderophore alcaligin (17, 51), the presence of a ton locus in its genome suggests that iron uptake is mediated by other Ton-dependent systems in *B. avium*.

**Cloning and sequencing of the tonB upstream region.** To localize the tonB promoter, we first isolated the tonB upstream region from *B. bronchiseptica* by using the strategy described in Materials and Methods. The 2.4-kb EcoRI fragment located immediately upstream from tonB was cloned into pBCSK + to obtain pEP532 (Fig. 1C). Sequence analysis of this insert revealed the presence of three ORFs oriented in the same direction as the tonB gene (Fig. 1C). Databases were scanned for similarities to the deduced amino acid sequences. The first ORF translates into a protein presenting 34% of identity in a 181-aa overlap with the C-terminal domain of MetY, an O-acetylhomoserine sulfhydrylase involved in methionine synthesis in *Leptospira meyeri* (7). The second ORF, starting 41 bp downstream from the metY termination codon, encodes a 226-aa protein homologous to PiuC, a putative *P. aeruginosa* iron uptake factor (65% identity in a 226-aa overlap) (47). No putative transcription terminator could be identified downstream from *B. bronchiseptica* piuC. A 322-aa noncoding region separates this ORF from the next one. The third ORF, hupB, codes for the 90-aa subunit of a putative histidine-protein kinase HU (71% identity with HU-B from *E. coli*). A 12-bp inverted repeat, AGGCCAATTCGCCGTCGCAGTTTGCCT, located 9 bp downstream from the hupB termination codon could form a transcriptional termination signal. Another inverted repeat GCCGCCCTCCGCCGTCGCTGGCGAGCGGCC is present 294 bp downstream from hupB. No similarity with any sequence in the databases could be detected in the 423-bp sequence downstream from hupB. Based on the *B. bronchiseptica* piuC and *B. pertussis* tonB sequences, we designed primers to PCR amplify the *B. pertussis* tonB upstream region. Sequence analysis of the PCR product indicated that this region is identical in both species.

The presence of potential FBS in pEP532 was tested in the Fur titration assay (58). *E. coli* H1717(pEP532) presented a Lac + phenotype, indicating the presence of at least one FBS on the EcoRI fragment (Fig. 1C). A derivative containing only the 423-bp HincII-EcoRI hupB downstream region also conferred a Lac + phenotype in the assay. The EcoRI-SalI fragment containing the metY and the piuC 5′-region was negative in the FBS assay (Fig. 1B), suggesting that, contrary to *P. aeruginosa*, the *B. bronchiseptica* piuC promoter does not contain any FBS. The HincII-EcoRI fragment was scanned for sequence similarity with the *E. coli* Fur-binding consensus sequence GATAATGATAATCATCATTATC. A putative GAGCTTGCGA similarity with the *E. coli* Fur promoter sequences, this region most likely contains the tonB promoter.

A *B. pertussis* ΔtonB exbB mutant is affected in iron uptake. To construct a *B. pertussis* tonB null mutant, the tonB upstream region was first spliced to the Km 1 cassette of pEP491 as described in experimental procedures to yield pEP552 (Fig. 1B). This plasmid was then introduced into *B. pertussis* BPSM. Selection for Km 1 Sm 1 clones enabled us to isolate the ΔtonB exbB::1Km' BPEP98 mutant by allelic exchange. BPEP98 colonies were hemolytic on BG plates, indicating that the adenylate cyclase-hemolysin (AC-Hy) virulence factor was produced.

When BPSM and BPEP98 were grown to stationary phase in SS or SS-Fe medium, no difference in growth rate or in final yield could be detected between the two strains (data not shown). This suggests that traces of iron in SS-Fe medium were sufficient to feed BPEP98. The culture supernatants were tested for siderophore activity in the CAS assay (56). The levels of siderophore activity were similar for both strains (data not shown). No siderophore activity was detected in the iron-replete culture supernatants of either strain. Whole-cell lysates (WCLs) were subjected to SDS-polyacrylamide gel electrophoresis (FAGE) analysis. No difference in the protein profiles of BPEP98 and BPSM was observed. Furthermore, both strains presented the same pattern of iron-repressed and iron-induced proteins (data not shown), indicating that the tonB mutant is still Fur regulated.

We next compared the growth of BPSM and BPEP98 at different concentrations of the Fe(III) chelator ethylenediamine di(o-hydroxyphenylacetic acid) (EDDHA) in SS-Fe media. For unknown reasons *B. pertussis* did not grow in liquid media even with very low concentrations of EDDHA. We therefore investigated their growth on solid SS media. BPSM and BPEP98 were first cultivated on BG plates and then streaked onto SS-Fe–0.1% Casamino Acids agarose plates with or without addition of 10 μM FeSO 4. The diameter of isolated colonies was measured after incubation for 6 days at 37°C. On SS plus Fe plates, both BPEP98 and BPSM formed ca. 1-mm-wide colonies. However, on SS-Fe plates, BPEP98 formed pinpoint colonies of about only 0.25 mm in diameter, compared to ca. 1 mm for the parental strain. Furthermore, in contrast to BPSM, the tonB mutant was not able to grow on SS-Fe plates containing 5 μM EDDHA (data not shown). These results suggest that BPEP98 is deficient in iron uptake.

The isogenic pair was tested for its ability to use different iron sources. SS-Fe plates containing 10 μM EDDHA were seeded with BPSM or BPEP98. Wells were punched in the agarose and filled with 15 μM solutions of FeSO 4, FeCl 3, or Fe(III)-loaded molecules such as hemin or deferasvir and ferrichrome siderophores. The plates were then incubated for 24 h at 37°C, after which the diameters of growth halos around the wells were measured. BPSM was able to grow using all five iron sources tested, whereas none of them promoted growth of the tonB mutant (Table 2). BPEP98 grew only around wells filled with 1 mM FeSO 4 or FeCl 3 (data not shown). Iron uptake of the avirulent *B. pertussis* ΔbvgAS mutant BLOW was tested under the same conditions. Similar to BPSM, BLOW could use all five iron sources (Table 2), indicating that the iron uptake systems involved are not Bvg dependent.

In *E. coli*, the antibiotic albomycin is transported into the cell via the ferrichrome receptor and the Ton system; thus, tonB mutants are albomycin resistant. BPSM, BPEP98, and BLOW were therefore tested for sensitivity to albomycin. As indicated in Table 2, BPSM and BLOW were sensitive to albomycin, while the tonB mutant was totally resistant.
Iron uptake is restored by the integration of a tonB exbBD operon copy into the ΔtonB exbB mutant chromosome. In order to complement the ΔtonB exbB mutation, we first cloned the B. pertussis tonB exbBD operon into a Bordetella multicopy plasmid. However, the introduction of this construct into BPEP98 or BPSM greatly reduced the viability of both strains, perhaps due to overproduction of the Ton system (data not shown). Thus, we reconstituted the ΔtonB exbB tonB exbB(Bs) locus on a Bordetella suicide plasmid to obtain pEP636 as described in Materials and Methods. This plasmid was then introduced into BPSM and BPEP98 by conjugation. Selection for Gnr Smr clones enabled us to isolate BPEP269 and BPEP270 bearing pEP636 inserted on the chromosome in the tonB region. Both strains were able to grow on SS-Fe plates containing 5 μM EDDHA. Furthermore, as shown in Table 2, BPEP270 was able to utilize all iron sources tested and was albomycin sensitive. This phenotype indicated complementation of the ΔtonB exbB mutation.

The Ton system is required for efficient colonization in the mouse model. To test whether the Ton system is required for virulence, mice were infected with either BPSM or BPEP98, and bacteria in the lungs were numerated at different time intervals after infection. As shown in Fig. 3, the parental strain was able to adhere and multiply in the lungs. Then, 1 week after infection, the number of bacteria declined. The ΔtonB exbB mutant behavior was different; it was unable to multiply during the first phase of the infection, but it was cleared at a rate similar to that of the parental strain. Thus, BPEP98 is affected in its capacity to multiply in the respiratory tract of the mouse.

The ΔtonB exbB mutant produces virulence factors and is sensitive to modulation signals. The colonization of the mouse respiratory tract depends on the production of B. pertussis adhesins and toxins, such as filamentous hemagglutinin (FHA), pertactin (PRN), pertussis toxin (PTX), and AC-Hly (for a review, see reference 39). The production of these virulence factors is controlled by the two-component regulatory system BvgAS, which undergoes phenotypic modulation in response to MgSO4 or nicotinic acid. To investigate whether the tonB mutation affects the production of the Bvg-dependent virulence factors or modifies Bvg regulation, WCLs and culture supernatants of BPSM and BPEP98 grown in SS, SS plus MgSO4, or SS plus nicotinic acid were compared by SDS-PAGE and immunoblot analyses. Both strains were found to produce similar amounts of FHA, AC-Hly, PRN, and PTX (data not shown). Protein profiles of BPSM or BPEP98 grown in modulation conditions were identical (data not shown), indicating that BPEP98 is responsive to chemical modulators. These observations imply that TonB is not required for virulence factor production or for modulation.

TonB production is independent of the BvgA and AlcR activators. To determine whether tonB is Bvg or AlcR regulated, the presence of TonB was examined in B. pertussis bvgAS or alcR null mutants. WCLs of B. pertussis BPSM, BPEP98 (ΔtonB exbB), BPEP184 (alcR::Km'), BLOW (ΔbvgAS), and wild-type B. bronchiseptica and B. parapertussis strains were analyzed by immunoblotting using MAb 4H4 raised against E. coli TonB (34). As shown in Fig. 4, an immunoreactive protein was detected in all Bordetella extracts (lanes 5 to 9) except for BPEP98 (lane 4). This protein had an apparent MW of 42,000 compared to about 38,000 for E. coli TonB (lane 1) and presented the same migration profile as B. pertussis TonB overproduced in E. coli (lane 3). TonB proteins exhibit a retarded migration due to their high proline content. Some minor retractive polypeptides in lanes 5 to 9 correspond probably to B. pertussis TonB degradation products since they were not observed in the BPEP98 WCL (lane 4) but were detected in E. coli overproducing B. pertussis TonB. These results show that (i) B. pertussis, B. bronchiseptica, and B. parapertussis synthesize a TonB homologue; that (ii) this protein is absent in B. pertussis ΔtonB exbB, and that (iii) B. pertussis TonB production does not require the BvgAS system or AlcR.
**DISCUSSION**

In this study we report the identification and functional characterization of the *B. pertussis* tonB exbB exbD locus. In light of their tight organization, tonB, exbB, and exbD are probably transcribed as a single operon. A similar gene arrangement has been documented for the Ton systems of *Neisseria meningitidis* (59), *H. ducreyi* (19), *P. putida* (23), and the first set of *Vibrio cholerae* tonB genes (46). In other species, such as *P. putida* (9), *H. influenzae* (26), *H. ducreyi* (19), *Pasteurella haemolytica* (25), Helicobacter pylori (61), and in the second tonB locus of *V. cholerae*, genes are clustered in the order exbB exbD tonB. In Enterobacteriaceae, tonB is not linked to the exbB exbD genes on the chromosome (15, 16, 22, 25). The *E. coli* tonB promoter has been shown to be Fur repressed (49), and exbB exbD are cotranscribed from an iron-regulated promoter (1). We identified an FBS upstream from tonB, suggesting that expression of the *B. pertussis* tonB exbBD operon is also derepressed in low-iron growth conditions.

The deduced *B. pertussis* TonB sequence presents the highest degree of similarity with that of *P. aeruginosa* TonB (48), which is in agreement with the phylogenetic proximity of these two species. A second tonB gene was recently identified in *P. aeruginosa* through sequence analysis of the *Pseudomonas* Genome Project, but its physiological role has not been identified yet (67). This tonB2 gene precedes putative exbB and exbD genes on the *P. aeruginosa* chromosome, unlike tonB1, which is not linked to potential exb genes (E. Pradel, personal observation). Two sets of *tonB* exbB exbD genes have been identified in *V. cholerae*, and both of them are involved in iron uptake (46). We used the Bordetella BLAST server of the Sanger Centre to scan the available *B. pertussis* genomic DNA sequences for similarities with *tonB*, *exbB*, and *exbD*. A unique *tonB* exbBD locus was detected in the 543 assembled contigs which cover most of the *B. pertussis* genome. On a distinct contig, we identified two linked ORFs encoding similar proteins to both *B. pertussis* ExbB and ExbD (27 and 35% conserved residues, respectively). However, the deduced proteins showed a higher degree of sequence similarity with *P. aeruginosa* TolQ and TolR (52 and 38% identity, respectively) (data not shown). No second ORF similar to *tonB* was detected. This analysis suggests that *B. pertussis* possesses a unique *tonB* exbBD operon and potential tolQR genes. The tolQR genes were not detected in our Southern hybridization experiments on *B. pertussis* chromosomal DNA, probably due to insufficient sequence conservation with the *exbBD* probe.

The *B. pertussis* TonB protein was overproduced in *E. coli*. Recombinant *B. pertussis* TonB was recognized by MAb 4H4 directed against *E. coli* TonB. This MAb has been shown to bind to the proline-rich region of *E. coli* TonB and to react with a unique protein in WCLs of a wide range of gram-negative species (34). However, 4H4 recognizes two putative TonB proteins in *P. aeruginosa* WCLs (34). A single protein presenting an electrophoretic migration similar to that of recombinant *B. pertussis* TonB was immunodetected in WCLs of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. This protein was absent from the WCL of a *B. pertussis* ΔtonB exbB::Km' mutant. Together, these observations suggest that *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis* produce only one TonB protein.

We showed that a *B. pertussis* ΔtonB exbB::Km' mutant is more sensitive to iron deprivation than the parental strain, while it is still able to synthesize and secrete alcAlcaligin in low-iron growth conditions. Brickman and Armstrong recently characterized FauA, the *B. pertussis* and *B. bronchiseptica* Alcaligin receptor, and suggested that FauA is TonB dependent based on its primary structure (14). The reduced growth of the ΔtonB exbB strain in iron-restricted medium could result from its inability to transfer the ferrialcaligin complex to the periplasm in the absence of TonB. Furthermore, the mutant is unable to use exogenous siderophores or hemin as sole iron source and to internalize the antibiotic albomycin, a ferrichrome structural analogue. Three additional outer membrane siderophore receptors have been characterized in *B. pertussis*, and analysis of their primary structure suggested that these proteins are TonB dependent (3, 5). Although no *B. pertussis* receptor for ferrichrome, desferal (ferrioxamine B), or hemin has been identified yet, our data indicate that these iron uptake systems are also TonB dependent.

*E. coli* tonB mutants are relatively iron starved, and genes normally regulated by Fur are derepressed even in high-iron conditions (50). We observed no difference in the iron-regulated protein profiles of the *B. pertussis* ΔtonB exbB::Km' and parental strains. Most likely, Fe(II) diffusion through porins is sufficient to maintain Fur repression in the mutant grown in iron-rich medium (36 μM FeSO₄). Transport of periplasmic Fe(II) into the cell is TonB independent and may occur via a cytoplasmic membrane protein similar to the Feo system in *E. coli* (12). We also showed that the production of the major *B. pertussis* virulence factors, such as FHA, PRN, PTX, and AC-Hly, does not require TonB. In addition, phenotypic modulation in response to chemical stimuli occurs in the absence of the Ton system; thus, the BvgAS virulence regulatory system is TonB independent. Conversely, we established that the production of TonB in *B. pertussis* is BvgAS independent, which is consistent with our observation that ferrichrome, desferal, or hemin usage as iron sources is not affected in a ΔbvgAS mutant. Thus, tonB is not part of the bvg regulon. In addition, tonB is also not regulated by AlcR, the transcriptional activator of alcAlcaligin biosynthesis and receptor genes (6, 51).

We had previously reported that a *B. pertussis* alcR mutant, while unable to produce alcAlcaligin, is not impaired in a murine respiratory infection model (51). In the present study we demonstrate that the ΔtonB exbB mutant is affected in its capability to multiply in the mouse respiratory tract. This observation suggests that TonB-dependent iron uptake systems are required for efficient proliferation in vivo. Involvement of TonB in virulence expression in animal models in relation with iron uptake capability has been documented previously for *H. influenzae* (29), *V. cholerae* (27), and *Salmonella typhimurium* (64). We cannot discard the hypothesis that the *B. pertussis* TonB function may not be restricted to iron uptake. Recently, *P. aeruginosa* TonB has been shown to play a role in efflux-mediated multidrug resistance (67). We can therefore not exclude that in *B. pertussis*, the Ton system could be involved in the transport of other substrates or in the expression of yet-identified virulence factors in the host.

**ACKNOWLEDGMENTS**

We thank Kathleen Postle for her encouragement and the gift of anti-TonB Mabs, Robert Kadner and Dominique Raze for the gift of plasmids and strains, and Klaus Hanke and Hans-Peter Fiedler for that of albomycin. We are grateful to Eve Willery, Sabine Thiberge, and Claudie Gantiez for technical assistance; to Emmanuelle Fort for photographic work; and to Franck Biet and Alain Baulard for computer counseling. We acknowledge Tejin for the supply of dimethyl β-maltodextrin, and Ciba-Gey for that of desferal. This work was supported by INSERM, the Institut Pasteur de Lille, the Région Nord-Pas-de-Calais, the Fondation de l’Institut Pasteur (Paris), and the Ministère de l’Éducation Nationale, de la Recherche, et de la Technologie.
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