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Bordetella pertussis TonB, a Bvg-Independent Virulence Determinant

ELIZABETH PRADEL,[1] NICOLE GUISO,[2] FRANCO D. MENOZZI,[1] AND CAMILLE LOCHT[1]*

INSERM U447, Institut Pasteur de Lille, 59019 Lille Cedex,[1] and Laboratoire des Bordetella, Institut Pasteur, 75724 Paris Cedex 15,[2] France

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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 HU and of a new two-component regulatory system were identified upstream from *tonB* and downstream from *exbD*, respectively. A *B. pertussis* Δ*tonB exbB*::Km^r 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 Δ*tonB 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 B_{12}, group B colicins, 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 protonmotive 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* Δ*tonB 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*.

* Corresponding author. Mailing address: INSERM U447, Institut Pasteur de Lille, 1 rue du Prof. Calmette, BP 245, 59019 Lille Cedex, France. Phone: 33 (0) 3-20-87-11-51. Fax: 33 (0) 3-20-87-11-58. E-mail: Camille.Locht@pasteur-lille.fr.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant features[a]	Source or reference
Strains		
E. coli		
RK5048	*metE70 tonB*	26
H1717	*aroB fhuF*::λ *plac*Mu; Km^r	58
XL1-Blue	High efficiency transformation; Tc^r	Stratagene
SM10	Mobilizing strain; Km^r	57
BL21(DE3)/pLysS	High-stringency expression host; Cm^r	Novagen
B. avium 103004	Sm^r	Institut Pasteur, Paris, France
B. bronchiseptica BB1015	Sm^r but not *rpsL*	51
B. parapertussis PEP	Sm^r	45
B. pertussis		
BPSM	Derivative of Tohama I *rpsL*; Sm^r Nal^r	41
BPEP98	Derivative of BPSM but Δ*tonB exbB*::ΩKm^r	This study
BPLOW	Derivative of BPSM but Δ*bvgAS*	E. Willery, Lille, France
BPEP184	Derivative of BPSM but *alcR*::Km^r	51
BPEP269	Derivative of BPSM with pEP636 integration	This study
BPEP270	Derivative of BPEP98 with pEP636 integration	This study
Plasmids		
pSUTonBExbBD	pSU19 containing *E. coli tonB*, *exbB*, and *exbD*; Cm^r	R. Kadner
pCG475	pUC18 bearing BPSM *tonB exbB exbD basR* on a 3.1-kb *Eco*RI fragment; Ap^r	This study
pEP487	pCG475 with a *Bam*HI ΩKm^r cassette inserted in the *exbB Bcl*I site; Ap^r Km^r	This study
pHP45Ω-Km	Source of ΩKm^r cassette	20
$pBCSK^+$	High-copy-number vector; Cm^r	Stratagene
pEP498	$pBSCK^+$ bearing BPSM *tonB exbB exbD basR′* on a 3-kb *Eco*RI-*Xho*I fragment; Cm^r	This study
pEP532	$pBSCK^+$ bearing the BB1015 *tonB* upstream region, (*′metYpiuChupB*) on a 2.4-kb *Eco*RI fragment; Cm^r	This study
pJQ200mp18rpsL	*Bordetella* suicide vector, contains *E. coli rpsL*; Gn^r	D. Raze, Little, France
pEP491	pJQ200mp18rpsL bearing *tonB exbB*::ΩKm^r*exbB basR* on an *Eco*RI fragment isolated from pEP487; Gn^r Km^r	This study
pEP549	Derivative of pEP491 through *Sal*I deletion, bears ΩKm^r*exbD basR*; Gn^r Km^r	This study
pEP552	Derivative of pEP549 through insertion of a 1.3-kb DNA region 5′ of *tonB* on the chromosome; Gn^r Km^r	This study
$pET24a^+$	T7 promoter expression vector; Km^r	Novagen
pEP583	$pET24a^+$ derivative to produce *Bp* TonB; Km^r	This study
pJQ200mp18	*Bordetella* suicide vctor; Gn^r	52
pEP636	pJQ200mp18 bearing *′piuC hupB tonB exbBD basR′* on a 4.5-kb *Xba*I-*Xho*I fragment; Gn^r	This study

[a] Ap^r, Cm^r, Gn^r, Km^r, Nal^r, Sm^r, and Tc^r, 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* H1717 transformants was tested on MacConkey lactose agar plates containing 50 μM $FeCl_3$. *Bordetella* strains were grown 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 Na–L-glutamate · H_2O, 0.24 g of L-proline, 2.5 g of NaCl, 0.5 g of KH_2PO_4, 0.2 g of KCl, 0.1 g of $MgCl_2 \cdot 6H_2O$, 20 mg of $CaCl_2 \cdot 2H_2O$, 1.5 g of Tris, 10 g of Casamino Acids, 1 g of dimethyl β-cyclodextrin (a gift from Teijin, Japan), 40 mg of L-cystein, 4 mg of nicotinic acid, 0.4 g of ascorbic acid, 0.15 g of glutathione, and 10 mg of $FeSO_4 \cdot 7H_2O$. Low-iron medium was SS without addition of $FeSO_4 \cdot 7H_2O$ (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 $MgSO_4$ or 15 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 (Gn), 10 μg/ml; kanamicin (Km), 30 μg/ml; nalidixic acid (Nal), 30 μg/ml; streptomicin (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 $Vent_R$ 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 Génétique 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* partial genomic DNA library we had previously constructed in pUC18. The *Bam*HI ΩKm^r cassette was isolated from pHP45Ω-Km and inserted into the unique *Bcl*I site in pCG475. The resulting plasmid, pEP487, was digested with *Eco*RI, and the 5.3-kb fragment bearing *tonB exbB*::ΩKm^r *exbD basR* was cloned into the *Bordetella* suicide vector pJQ200mp18rpsL (Gn^r) 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 Gn^r Km^r BB1015 mutant bearing pEP491 inserted into the *tonB* locus was digested with *Nsi*I, 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 *Nsi*I fragment containing pEP491 was isolated. Restriction mapping of this plasmid permitted identification of the *B. bronchiseptica tonB* 5′ region. A 2.4-kb *Eco*RI DNA fragment localized immediately upstream from the *tonB* locus was subcloned into $pBCSK^+$ to yield pEP532 (Fig. 1C).

Construction of the *B. pertussis* Δ*tonB exbB*::ΩKm^r mutant. To delete *tonB* and *exbB* in pEP491, this plasmid was digested with *Sal*I and religated to yield pEP549 (Fig. 1B). Oligonucleotides T13 (5′-GCCAGTTGTCCGAGCAGCAC-

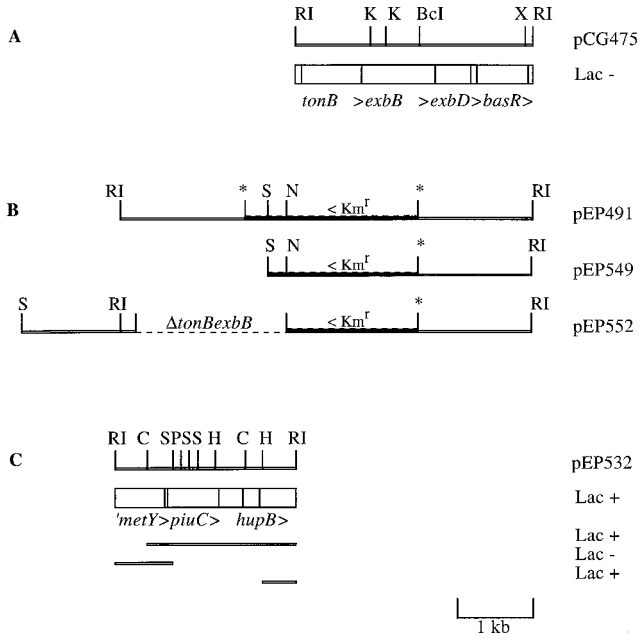

FIG. 1. Physical map of the *Bordetella tonB exbB exbD* chromosomal region and relevant constructions described in this study. The recombinant plasmid-associated Lac phenotypes in the Fur titration assay are indicated on the right. Blocks representing ORFs are drawn to scale; arrowheads indicate the direction of transcription. Certain restriction sites are indicated as follows: *Bcl*I (BcI), *Cla*I (C), *Eco*RI (RI), *Hin*cII (H), *Kpn*I (K), *Nru*I (N), *Pst*I (P), *Sal*I (S), and *Xho*I (X). (A) The *B. pertussis tonB exbB exbD* locus cloned as a 3.1-kb *Eco*RI fragment in pCG475. (B) Inserts in *Bordetella* suicide plasmids pEP491, pEP549, and pEP552. pEP552 was used for allelic exchange to construct the *B. pertussis* Δ*tonB exbB*::ΩKm^R mutant BPEP98. (C) The *B. bronchiseptica tonB* upstream region subcloned as a 2.4-kb *Eco*RI fragment in pEP532. Derivatives tested in the Fur titration assay are indicated.

3′), which hybridizes between the first *Sal*I site and the *Pst*I site in *piuC*, and T14 (5′-CACGACGAAGCGGCATTCCTA-3′), which hybridizes 39 bp downstream from the *Eco*RI site preceding *tonB*, were used to amplify the *tonB* upstream region from *B. pertussis* BPSM genomic DNA. PCR conditions were 35 cycles of 1 min of denaturation at 96°C, 1 min of hybridization at 60°C, and 1.5 min of elongation at 72°C. The 1.6-kb PCR product containing the *B. pertussis tonB* upstream region (*'piuChupB*) was digested with *Sal*I, and the resulting 1.3-kb fragment, which bore a *Sal*I and a blunt-ended extremity, was inserted into pEP549 opened with *Sal*I and *Nru*I to generate pEP552 (Fig. 1B). The *Nru*I site in pEP549 is located downstream of the Km^r gene; thus, pEP552 still confers resistance to Km. *E. coli* SM10 was transformed with pEP552 and used as a donor in conjugation with *B. pertussis* BPSM. Exconjugants were selected on BG-Nal-Km plates. All 27 isolated colonies tested showed a Sm^r Km^r Gn^s double recombination phenotype. Genomic DNA of two clones was prepared and subjected to Southern blot hybridization with the 1.9-kb *Kpn*I-*Eco*RI fragment of pCG475 carrying *exbB exbD*. Correct allelic exchange was confirmed in both strains. One of these Δ*tonB exbB*::ΩKm^r mutants, BPEP98, was chosen for further study.

Construction of BPEP269 and BPEP270. Plasmid pEP498 was digested with *Pst*I and *Eco*RI and ligated with the 1.5-kb *Pst*I-*Eco*RI fragment from pEP532 to reconstitute the *'piuC hupB tonB exbBD basR'* locus in pBCSK$^+$. Taking advantage of the *Xba*I site present in the multiple cloning site, the 4.5-kb *Xba*I-*Xho*I fragment was then cloned into pJQ200mp18 opened with *Xba*I and *Sal*I to yield pEP636. This Gn^r *Bordetella* suicide plasmid was introduced into BPSM and BPEP98 by conjugation with *E. coli* SM10(pEP636). Exconjugants were selected on BG-Sm-Gn. Two derivatives of BPSM and BPEP98 bearing pEP636 integrated on the chromosome, BPEP269 and BPEP270, respectively, were tested for iron source utilization and albomycin sensitivity.

Siderophore detection. The Chrome Azurol S (CAS) assay (56) was used to assess alcaligin production by *Bordetella* cells grown to stationary phase in iron-limited (SS-Fe) medium. A 0.5-ml volume of culture supernatant was added to 0.5 ml of CAS solution, and the A630 of the CAS dye was measured after incubation for 4 h at room temperature.

Iron source utilization and albomycin sensitivity tests. Fresh *B. pertussis* cells were scraped from BG plates and diluted into SS-Fe to an optical density at 600 nm (OD_{600}) of 1. A 200-μl aliquot of this suspension was added to 20 ml of 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 $FeSO_4$, $FeCl_3$, 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. Diameters of growth zones around wells were measured after 24 h of incubation at 37°C. To test albomycin sensitivity, 20 ml 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. Hantke 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 2×10^6 bacteria. Infected mice were sacrificed by cervical dislocation 2 h after exposure 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 on BG. To assess stability of the Δ*tonB exbB*::ΩKm^r mutation, bacteria reisolated from the lungs of BPEP98-infected mice were tested for their resistance to Km 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 *Nde*I-TonB (5′-ATATCATATGCCTAGCCCCCAATCTGGT-3′) and TonB-*Eco*RI (5′-ATATGAATTCTGGCGGTGTTGATAGCGTTG-3′) by 35 PCR cycles of 1 min of denaturation at 96°C, 1 min of hybridization at 63°C, and 1 min of elongation at 72°C. The amplification product was digested with *Nde*I and *Eco*RI and cloned into pET24a$^+$ to obtain pEP583. BL21(DE3)(pLysS) was transformed with either pET24a$^+$ or pEP583 and grown in LB-Km-Cm at 37°C. At an OD_{600} 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).

Immunoblotting. *E. coli* strains were grown in LB to an OD_{600} of 0.6, and then 1 ml of culture was precipitated at 4°C with one-third volume of 30% TCA, pelleted, washed with 50 mM Tris-HCl (pH 8.0), and then solubilized at 95°C for 5 min in 200 μl of Laemmli buffer (32). *Bordetella* strains were grown in SS to an OD_{600} of 3, and then a culture volume corresponding to 6 OD_{600} units was TCA precipitated (1 OD_{600} unit is equivalent to 1 ml of culture at an OD_{600} of 1), washed, and solubilized as described above for *E. coli*. Aliquots [15 μl for RK5048(pSUTonBExbBD), 5 μl for BL21(DE3)(pLysS) containing pET24a$^+$ or pEP583, and 20 μl for *Bordetella* strains] were resolved by electrophoresis on sodium dodecyl sulfate (SDS)–11% polyacrylamide gels. Proteins were then electrotransfered to Immobilon-P membranes (Millipore, St-Quentin-en-Yvelines, France), probed with a 1:5,000 dilution of murine monoclonal antibody (MAb) 4H4 raised against *E. coli* TonB (a gift from K. Postle) (34), and developed by colorimetric detection with alkaline phosphatase-conjugated secondary antibodies.

Nucleotide sequence accession numbers. Sequence data were submitted to the EMBL database under accession nos. AJ132741 and AJ132742.

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 *B. pertussis* 3.1-kb *Eco*RI 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 *basR*. The putative *tonB* ATG start codon is located 80 bp downstream from the *Eco*RI site. It is not preceded by a sequence resembling the canonical *E. coli* ribosome binding site; this is often observed with *B. pertussis* genes. The putative *exbB* ATG initiation codon at position 882 is separated from the *tonB* TGA stop codon by 1 bp, and the putative *exbD* ATG start codon at position 1859 overlaps the *exbB* TAA termination codon. Such a tight organization suggests that the three genes are cotranscribed. Most *tonB* genes contain a Fur-binding sequence (FBS) in their promoter region, and their expression is repressed by

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 *Eco*RI-*Xho*I *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 *Eco*RI-*Xho*I 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 (GCCCGCGCCGCGGGCGCGGGC) 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 *Eco*RI 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 266-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). *B. pertussis* TonB, *P. aeruginosa* TonB, and *E. coli* TonB contain 20, 18, and 17% Pro, respectively; and (iii) a PXYP motif and highly conserved Val and Gly residues are present in the C-terminal third of the protein (Fig. 2A). In *E. coli* TonB 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, compared to 25% identity with *E. coli* ExbB in a 257-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 $VX_3L/VX_3LX_3SX_3W$ 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

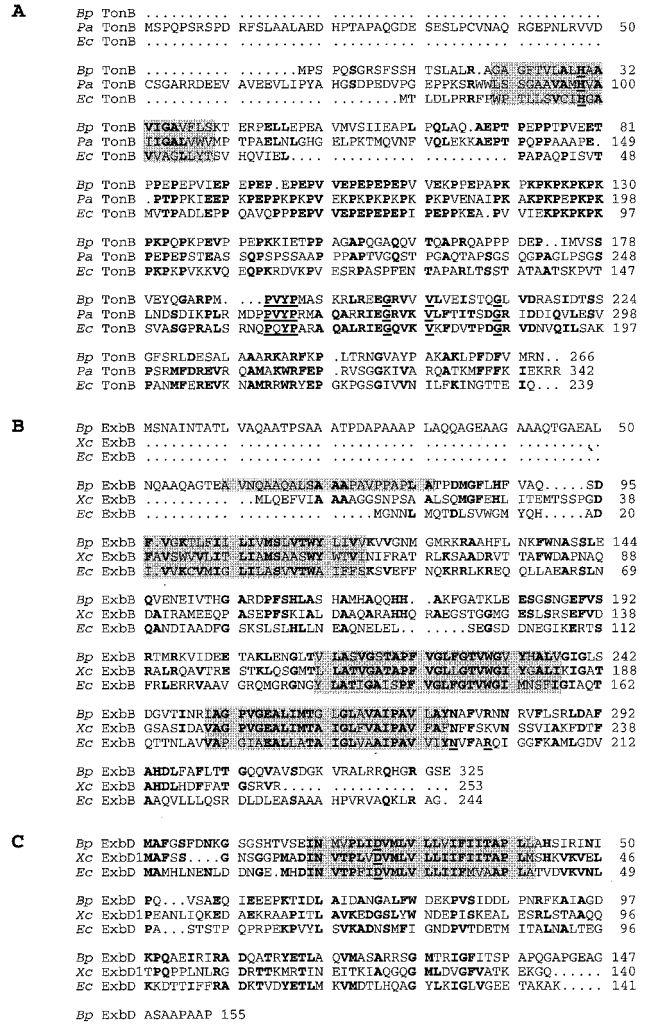

FIG. 2. Sequence alignments of the deduced *B. pertussis* (*Bp*) TonB (A), ExbB (B), and ExbD (C) proteins with their closest homologues and the corresponding *E. coli* (*Ec*) proteins. *Pa*, *P. aeruginosa*; *Xc*, *X. campestris*. Predicted transmembrane segments are highlighted in gray. Conserved residues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

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 *hupB-tonB* 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: 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 V120A. However, due to a 33-bp in-frame deletion, the 19385 ExbB contains only one of the two GTEAV/LNQAAQA motifs present in BPSM ExbB N-terminal extension. Comparing the 19385 and BPSM *exbD* genes, we noticed two changes

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 *Eco*RI and probed with the 3-kb *Eco*RI-*Xho*I DNA fragment of pCG475 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 *Eco*RI 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 *Eco*RI 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 product 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-bp noncoding region separates this ORF from the next one. The third ORF, *hupB*, codes for the 90-aa β-subunit of a putative histone-like protein HU (71% identity with HU-β from *E. coli*). A 12-bp inverted repeat, AGGCAAATCGGCGCTGCCGATTTGCCT, located 9 bp downstream from the *hupB* termination codon could form a transcriptional termination signal. Another inverted repeat GCGCGCTCGGCGCGTGCCGAGCGCGC 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 *Eco*RI fragment (Fig. 1C). A derivative containing only the 423-bp *Hinc*II-*Eco*RI *hupB* downstream region also conferred a Lac$^+$ phenotype in the assay. The *Eco*RI-*Sal*I fragment containing *′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 *Hinc*II-*Eco*RI fragment was scanned for sequence similarity with the *E. coli* Fur-binding consensus sequence GATAATGATAATCATTATC. A putative GAGCTTGCGA ATCATTCGC FBS (12 of 19 matches) was identified 36 bp upstream from the *Eco*RI site. Since FBSs usually overlap promoter sequences, this region most likely contains the *tonB* promoter.

A *B. pertussis* Δ*tonB 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^r 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^r Sm^r clones enabled us to isolate the Δ*tonB exbB*::ΩKm^r BPEP98 mutant by allelic exchange. BPEP98 colonies were hemolytic on BG plates, indicating that the adenylate cyclase-hemolysin (AC-Hly) 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 (PAGE) 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 restreaked 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 desferal 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* Δ*bvgAS* mutant BPLOW was tested under the same conditions. Similar to BPSM, BPLOW 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 BPLOW were therefore tested for sensitivity to albomycin. As indicated in Table 2, BPSM and BPLOW were sensitive to albomycin, while the *tonB* mutant was totally resistant.

TABLE 2. Iron sources utilization and albomycin sensitivity of *B. pertussis* BPSM, BPEP98, BPLOW, BPEP269, and BPEP270[a]

Strain	Confluent growth halo diam (mm) with:						Albo[d]
	SS-Fe	$FeSO_4$	$FeCl_3$	Hemin	DF[b]	FC[c]	
BPSM	0	9	9	21	27	27	S
BPEP98	0	0	0	0	0	0	R
BPLOW	0	7	7	21	27	26	S
BPEP269	0	6	11	20	27	27	S
BPEP270	0	6	12	20	25	25	S

[a] The diameters of confluent growth halos around wells filled with SS-Fe or the indicated iron source (15 μM solution in SS-Fe) were measured in millimeters after 24 h at 37°C. Results are the means for two separate experiments.
[b] DF, desferal (ferrioxamin B).
[c] FC, ferrichrome.
[d] Sensitivity (S) or resistance (R) to albomycin.

Iron uptake is restored by the integration of a *tonB exbBD* operon copy into the Δ*tonB exbB* mutant chromosome. In order to complement the Δ*tonB 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 *'piuC hupB tonB exbBD basR'* 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 Gn^r Sm^r 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 Δ*tonB 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 Δ*tonB 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.

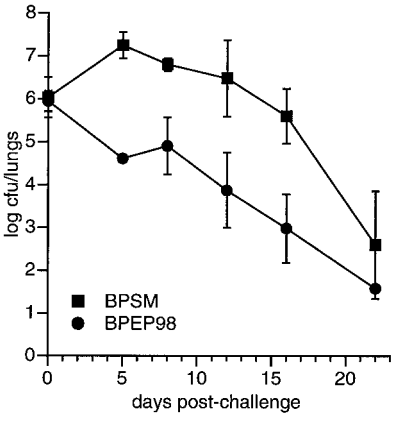

FIG. 3. Colonization profiles of parental BPSM and BPEP98 Δ*tonB exbB* in the murine respiratory infection model.

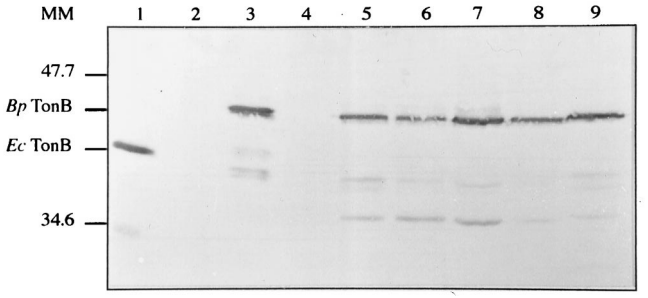

FIG. 4. TonB immunoblots of WCLs. Lanes 1 to 3, *E. coli* strains RK5048(pSUTonBExbBD), BL21(DE3)/pLysS(pET24a$^+$), and BL21(DE3)/pLysS(pEP583), respectively. Lanes 4 to 7, *B. pertussis* strains BPEP98 Δ*tonBexbB*, parental BPSM, BPEP184 *alcR*, and BPLOW Δ*bvgAS*, respectively. Lane 8, *B. bronchiseptica* BB1015. Lane 9, *B. parapertussis* PEP. The positions of *B. pertussis* TonB, *E. coli* TonB, and the molecular mass (MM) standards (in kilodaltons) are indicated on the left.

The Δ*tonB 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 $MgSO_4$ 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 $MgSO_4$, 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 (Δ*tonB exbB*), BPEP184 (*alcR*::Km^r), BPLOW (Δ*bvgAS*), 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 reactive 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 (lane 1). 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* Δ*tonB 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), *N. gonorrhoeae* (8), *X. campestris* (66) and for the first set of *Vibrio cholerae tonB* genes (46). In other species, such as *P. putida* (9), *H. influenzae* (28), *H. ducreyi* (19), *Pasteurella haemolytica* (23), *Helicobacter pylori* (61), and in the second *tonB* locus of *V. cholerae* (46), 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 products similar to *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* Δ*tonB exbB*::Km^r mutant. Together, these observations suggest that *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis* produce only one TonB protein.

We showed that a *B. pertussis* Δ*tonB exbB*::Km^r mutant is more sensitive to iron deprivation than the parental strain, while it is still able to synthesize and secrete alcaligin 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 Δ*tonB 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* Δ*tonB exbB*::Km^r 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 $FeSO_4$). 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 Δ*bvgAS* mutant. Thus, *tonB* is not part of the *bvg* regulon. In addition, *tonB* is also not regulated by AlcR, the transcriptional activator of alcaligin biosynthesis and receptor genes (6, 51).

We had previously reported that a *B. pertussis alcR* mutant, while unable to produce alcaligin, is not impaired in a murine respiratory infection model (51). In the present study we demonstrate that the Δ*tonB 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-unidentified virulence factors in the host.

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