Detection and Subcellular Localization of Three Ptl Proteins Involved in the Secretion of Pertussis Toxin from *Bordetella pertussis*

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The *ptl* locus of *Bordetella pertussis* contains eight open reading frames which are predicted to encode proteins (PtlA to PtlH) that are essential for secretion of pertussis toxin from the bacterium and which are members of a family of transport proteins found in other types of bacteria. We have detected PtlE, PtlF, and PtlG in immunoblots of extracts of *B. pertussis* by using antibodies raised to fusion proteins consisting of maltose-binding protein and the individual Ptl proteins. These proteins have apparent molecular weights similar to those predicted by DNA sequence analysis. Cell fractionation studies indicated that all three Ptl proteins are associated with the membranes of *B. pertussis*, suggesting that the Ptl proteins form a gate or channel which facilitates transport of pertussis toxin. Cell extracts of other *Bordetella* spp. were probed with antibodies to Ptl proteins for the presence of these transport proteins. Neither *Bordetella parapertussis* nor *Bordetella bronchiseptica* contained detectable levels of PtlE or PtlF. This lack of detectable Ptl protein may provide an explanation for previous observations which indicated that introduction of the genes encoding pertussis toxin subunits from *B. pertussis* into other *Bordetella* spp. results in production of the toxin but not secretion of the toxin.

Export of virulence factors such as toxins, adhesins, and invasins from pathogenic bacteria is often critical to the success of the pathogen, since exposure of these virulence factors optimizes their interaction with host cells. *Bordetella pertussis*, the causative agent of pertussis (whooping cough), secretes several virulence factors, one of which is pertussis toxin (PT), a toxin that may contribute to pathogenesis by impairing the immune system of the host (11). PT is an important virulence factor of *B. pertussis*, since mutant strains of *B. pertussis* which do not produce PT are less virulent in animal models than the wild-type strain (36). A strain of *B. pertussis* which produces PT but which is defective in the secretion of the toxin is also less virulent than the wild-type strain in animal models (35), suggesting that secretion of the toxin, not simply production of PT, is important for pathogenesis.

The question arises as to how PT might be transported across the inner and outer membranes of *B. pertussis*. The toxin is a large, complex protein composed of six subunits (S1, S2, S3, S4, and S5 found in a 1:1:1:2:1 ratio) and has a molecular weight of 105,000 (21, 27). Each subunit is synthesized with a signal sequence (18, 21), suggesting that the subunits may be transported into the periplasmic space via a general export pathway analogous to the *sec* system of *Escherichia coli* (39). Others have reported that the S1 subunit is required for efficient secretion of PT (23), suggesting that an assembled form of the molecule must be transported across at least one membrane of the bacterium.

Recently, we defined a region of the *B. pertussis* chromosome, termed the *ptl* locus, which is critical for efficient secretion of PT (37). This region, which spans approximately 9.5 kb, contains eight open reading frames (ORFs), termed orfA to orfH. Evidence suggests that the *ptl* genes are located within a single operon (37). Each of the eight ORFs is predicted to encode a protein homologous to one of the VirB proteins produced by *Agrobacterium tumefaciens* (6, 37). The VirB proteins are thought to be involved in the transport of a piece of single-stranded DNA, T-DNA, across bacterial membranes (13, 31, 32, 40). These homologies, together with the demonstration that mutations in the *ptl* locus affect PT secretion (37), suggest that the *ptl* genes encode proteins needed for transport of PT across the bacterial membrane(s).

While the existence of Ptl proteins is predicted, they have not as yet been detected. In this report, we describe the identification of three of the Ptl proteins, PtlE, PtlF, and PtlG, and their cellular localization. In addition, we used antibodies to the Ptl proteins to probe extracts of related *B. pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica* strains which are known to be defective in the secretion of PT for detection of the presence of Ptl proteins, and we offer an explanation for the lack of secretion of PT in these strains.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** *B. pertussis* BP338, BP356, BPPTL2, BPPTL3, BPPTL4, and BPM3171 were obtained from Alison Weiss, University of Cincinnati. *B. pertussis* Tohama III was from the collection of the Laboratory of Pertussis, Center for Biologies Evaluation and Research, Food and Drug Administration, Bethesda, Md. *B. pertussis* 18323 (ATCC 9797), *B. parapertussis* 9305, and *B. bronchiseptica* Bb55 (ATCC 31437) were from the American Type Culture Collection (Rockville, Md.). These strains were grown at 37°C on plates of Bordet-Gengou medium containing 1.25% glycerol and 15% defibrinated sheep blood (University Micro Reference Laboratory, Inc., Linthicum, Md.) either with or without 1.5% proteose peptone. The following antibiotics were added to the medium for the indicated strains: gentamicin, 10 μg/ml (strains BPPTL2, BPPTL3-2, and BPPTL4); streptomycin, 150 μg/ml (strain BPPTL3-2); and kanamycin, 25 μg/ml (strain BPM3171). Modulated *B. pertussis* cells were
harvested from media supplemented either with 50 mM MgSO₄ or with 10 mM nicotinic acid and 20 mM MgSO₄.

Library efficiency-competent cells, E. coli DH5α, were obtained from Gibco BRL (Gaithersburg, Md.), and the plasmid pMAL-c2 was purchased from New England Biolabs (Beverly, Mass.). Cells were grown on Luria-Bertani (LB) agar (Difco Laboratories, Detroit, Mich.) or in liquid culture containing SOC medium (GIBCO BRL) or LB broth supplemented where necessary with ampicillin (100 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 50 μg/ml), and/or isopropyl-β-D-thiogalactopyranoside (IPTG).

PCR. Reagents for DNA amplification were obtained from Gibco BRL. The oligonucleotide primers used were obtained from Lofstrand Labs Limited (Gaithersburg, Md.). For each PCR, the upstream primer contained three nucleotides followed by an XbaI site, the ATG initiation codon, and the next 19 nucleotides of the ORF at its 5′ end. The downstream primer (5′→3′) contained three nucleotides followed by a HindIII site, the termination codon and the last 19 nucleotides of the ORF. Two templates were used for PCRs. A plasmid (pUW1096C) obtained from Alison Weiss, University of Cincinnati, which contained nucleotides 4691 to 10840 of the pil locus was used for amplification of ORFs E and F. B. pertussis Tohama I DNA, kindly provided by Z. M. Li (Food and Drug Administration), was used for amplification of ORF G. Each PCR mixture contained two oligonucleotide primers (0.25 μM), template (either 30 ng of Tohama I DNA or 167 ng of pUW1096C), deoxynucleotides (each at 1 mM) and AmpliTaq DNA polymerase (2.5 U; Perkin-Elmer Corp., Norwalk, Conn.) in 10 mM Tris-HCl, pH 8.3, containing 50 mM KCl. The reaction mixture also contained MgCl₂ and glycerol at final concentrations of 2.5 mM and 20%, respectively. Amplified DNA was purified with the Magic PCR Prep DNA purification system (Promega, Madison, Wis.).

Production and purification of fusion proteins. The Protein Fusion and Purification System (New England Biolabs) was used to generate and purify fusion proteins composed of maltose-binding protein (MBP) and proteins encoded by pil ORFs. DNA manipulations and subsequent procedures were performed by following the manufacturer’s instructions and using standard procedures (25). Briefly, DNA which had been amplified by PCR and which corresponded to each pil ORF was inserted into pMAL-c2 vectors that had been digested with XbaI and HindIII. The resulting plasmids were introduced into E. coli DH5α. Cells were grown to approximately 2 × 10⁶ cells per ml (A₅₅₀nm ≈ 0.5) when production of fusion proteins consisting of MBP and proteins encoded by orfE, orfF or orfG was induced by the addition of IPTG (0.3 or 1 mM) for 2 h. Cells were harvested, suspended in 50 ml of 10 mM Tris-HCl, pH 7.4, containing 200 mM NaCl and 1 mM EDTA (column buffer), and sonicated, and the resulting suspension was centrifuged at 9,000 × g for 30 min. The supernatant was applied to a column containing amylose resin (20 ml) according to manufacturer’s instructions. The column was washed with 8 column volumes of column buffer. The fusion protein was eluted with column buffer containing maltose (10 mM). Fusion proteins were dialyzed extensively against phosphate-buffered saline (PBS) at 4°C and were stored frozen.

Purification determination. Protein concentration was determined by the method of Bradford (4), with ovalbumin as the standard.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (15), with 4 to 20% gradient polyacrylamide gels (Integrated Separation Systems, Natick, Mass.). Proteins were stained with Coomassie brilliant blue R250.

Polyclonal antibody production and monoclonal antibodies. MBP-ORF fusion protein was added to PBS containing gelatin (0.05%) to give a final concentration of 60 μg/ml. Alhydrogel [Al(OH)₃] (Superfos, A/S, Vedbaek, Denmark) was added to the buffer to give a 1:1 ratio (on a weight basis) of Alhydrogel to protein (fusion protein plus gelatin). Preparations were incubated for 1 h at room temperature with occasional stirring.

Five female NIH(S) mice were each injected intraperitoneally with 0.5 ml of the preparation containing 30 μg of fusion protein, MBP, or the Alhydrogel solution containing only 0.05% gelatin. Two and 4 weeks after the initial injection, mice were given a second and third 0.5-ml injection. Two weeks later, blood was collected from the tail vein of each mouse. Sera were prepared, pooled, and stored at −20°C.

Monoclonal antibody 3CX4 which recognizes the S1 subunit of pertussis toxin (12) was obtained from James Kenimer, Food and Drug Administration. Monoclonal antibody MO8-X3C which recognizes filamentous hemagglutinin (FHA) (17) was obtained from Michael Brennan, Food and Drug Administration.

Immunoblot analysis. Cell extracts and fractions were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose membranes overnight at 30 V in 25 mM Tris-192 mM glycine with 20% methanol. Nitrocellulose sheets were incubated for 1 h with 10% (wt/vol) skim milk in distilled water. Nitrocellulose strips were washed three times with PBS, pH 7.0, containing 0.05% Tween 20 (PBS-Tween) and were incubated for 2 h with sera containing antibodies to fusion proteins (used at 1:2,000 or 1:4,000 dilution). After three further washes with PBS-Tween, the strips were incubated for 1 h with goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad Laboratories, Richmond, Calif.) diluted 1:1,000 with PBS-Tween. The nitrocellulose strips were washed with PBS, and the bands were visualized by adding the color reagent (15 mg of 4-chloro-1-naphthol in 5 ml of methanol combined with 25 ml of PBS containing 0.018% hydrogen peroxide).

Preparation of cell extracts and fractionation of extracts. Bordetella cells grown on BG agar were suspended in PBS to give an A₅₅₀ of 2, centrifuged at 12,000 × g for 10 min, and suspended in PBS to the same optical density. The suspension (7 μl) was mixed with SDS-PAGE sample buffer (13 μl of a solution containing 2% SDS, 20% glycerol, 200 mM Tris base, 100 mM dithiothreitol, 0.01% bromphenol blue). In certain experiments, as indicated, 20 μl of the suspension was mixed with an equal volume of 20% trichloroacetic acid. After centrifugation, the precipitate was suspended in SDS-PAGE sample buffer. All samples were boiled for 2 min and then subjected to gel electrophoresis.

Cell fractionation was performed essentially as described previously (26). Briefly, cells grown on plates containing BG medium were harvested from the plates and suspended in PBS to an A₅₅₀ of 2. This mixture (10 ml) was frozen in a dry ice-ethanol bath, thawed in cold water, and sonicated (model W375; Heat Systems Ultrasonic Inc.) on 50% pulse mode and power setting 3 for 5 min on ice. The mixture was centrifuged for 10 min at 2,000 × g to remove unbroken cells. Supernatant (5 ml) was centrifuged for 1 h at 107,000 × g. The resulting supernatant, which contained cytosolic and periplasmic proteins, was collected. The pellet containing the membrane fraction was suspended in 5 ml of PBS. Portions (500 μl) of both the supernatant and pellet fractions were precipitated in an equal volume of 20% trichloroacetic acid. The precipitates were collected after centrifugation and suspended in 20 μl of SDS gel sample buffer and subjected to electrophoresis.

Detergent solubilization of the membrane fraction was
conducted essentially as described previously (26). The pellet obtained after centrifugation of the sonicated cell extract at 107,000 × g for 1 h (see above) was suspended in 2.5 ml of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing 2% Triton X-100 and 7.5 mM MgCl2. The preparation was incubated at room temperature for 30 min and was then centrifuged at 107,000 × g for 1 h. The pellet (Triton X-100-insoluble fraction) was suspended in 2.5 ml of PBS. SM-2 Bio-beads (0.7 g, Bio-Rad) were added to the Triton X-100-soluble fractions to remove detergent (9) that interfered with resolution of proteins during the subsequent electrophoresis procedure. The mixture was agitated overnight at 4°C. The sample was centrifuged for 10 min at approximately 2,000 × g, and the supernatant was collected. The Triton X-100-insoluble fraction (125 µl) and Triton X-100-soluble fraction (400 µl) were each added to equal volumes of 20% trichloroacetic acid. After centrifugation, the precipitates were solubilized in 20 µl of SDS gel sample buffer and subjected to electrophoresis. Similar results were obtained when the Triton X-100-soluble fraction was not treated with the Bio-beads to remove the detergent (data not shown).

RESULTS

Identification of Ptl proteins. As shown in Fig. 1, the pld locus contains eight ORFs. Antibodies to proteins predicted to be encoded by orfE, orfF, and orfG were made by the following method. DNA corresponding to each of the ORFs was amplified using PCR. These fragments were then each inserted into pMAL-c2 vectors. The resulting recombinant plasmids were each transformed into E. coli, and fusion proteins consisting of the MBP and the predicted Ptl protein were produced. Fusion proteins were purified by affinity chromatography with amylose resin. Each of the fusion protein preparations was then used to immunize mice to generate antibodies to the Ptl proteins.

Sera from mice immunized with the MBP-orfE fusion protein were used to probe extracts of virulent B. pertussis for the presence of PtlE. As shown in Fig. 2, immunoblot analysis of extracts of BP338 (wild-type, virulent strain) revealed that the MBP-orfE antiserum bound to several proteins including a protein of apparent molecular weight of 30,000. The predicted molecular weight of PtlE obtained by nucleotide sequence analysis of orfE is 31,010. In order to determine whether any of these proteins might be PtlE, we also examined extracts of BP338 which had been grown in the presence of MgSO4. Growth of B. pertussis in the presence of MgSO4 (known as modulating conditions) reversibly suppresses expression of genes encoding virulence factors which are under control of products of the bvg (Bordetella virulence genes) locus (10, 14, 38). Previously, pld genes have been shown to be bvg regulated (38) and therefore would not be expected to be expressed in an avirulent form of the organism or in a virulent form of the organism grown under modulating conditions. The protein having an apparent molecular weight of 30,000 was not ob-
served in this extract. In order to confirm the identity of the 30,000-Da species as PtlE, we examined cell extracts of strains of B. pertussis which have mutations in the pld locus. The locations of these mutations are shown in Fig. 1. Strain BPPTL3-2 was constructed by deletion of a 437-bp BamHI fragment in orfC and replacement of this fragment with a 4.0-kbp BamHI cassette containing the gentamicin resistance gene and the P-incompatibility origin-of-transfer region (37) and therefore would not be expected to produce PtlE. Strain BPM3171 was generated by insertion of a Tn5lac transposon into orfC (37, 38) and thus would not be expected to produce PtlE. Strains BPPTL2 and BPPTL4 were generated by homologous recombination of a plasmid containing portions of the pld locus on the chromosome such that the vector would interrupt the chromosome in orfG and orfH, respectively (37). These mutants would be expected to produce PtlE since the mutations occur downstream from pldE. The 30,000-Da protein was not observed in extracts of BPPTL3-2 or BPM3171 but was observed in extracts of BPPTL2 and BPPTL4, as would be expected if the 30,000-Da protein were PtlE. Sera from control mice which had been injected with either MBP or Alhydrogel alone did not bind to the 30,000-Da protein (data not shown).

Cell extracts from the wild-type and mutant strains of B. pertussis were also probed with sera from mice immunized with the MBP-orfF fusion protein. As shown in Fig. 3, a protein with an apparent molecular weight of 31,000 was seen in cell extracts from the virulent strain BP338 but not in the modulated strain. This protein was seen in cell extracts of BPPTL2 and BPPTL4 but not in extracts of BPPTL3-2 or BPM3171, which would be the expected observation if the 31,000-Da protein were PtlF. Nucleotide sequence analysis of orfF predicts that the mature form of PtlF would have a molecular weight of 27,541 (37).

Serum from mice immunized with MBP-orfG fusion protein was used to probe B. pertussis cell extracts in order to identify PtIG. As shown in Fig. 4, this serum bound to a number of proteins in cell extracts from all of the strains. The reason for this high background binding is unknown. Nonetheless, a protein band with an apparent molecular weight of 38,000 was observed in extracts from the virulent form of BP338 and BPPTL4 but not in extracts of an avirulent strain (Tohama III) or BPPTL3-2, BPM3171, or BPPTL2. (It should be noted that the band having an apparent molecular weight of 38,000 migrated only slightly above a second band of almost identical molecular weight such that the two proteins appeared as a
doublet. The lower band was present in extracts from all strains.) BPPTL3-2 and BPM3171 would not be expected to produce PtlG, whereas BPPTL2 would be predicted to produce a truncated form of this protein which may or may not be stable. A smaller form of the protein was not observed in extracts of BPPTL2. A second band which migrated as a 46,000-Da protein was present in the same samples as the 38,000-Da protein and may represent a larger form of PtlG. Two forms of VirB10, the PtlG homolog in *A. tumefaciens*, have been described (33).

Attempts were made to identify other predicted Ptl proteins (PtlA, PtlB, PtlC, PtlD, and PtlH) by similar methods. We were unable to isolate a fusion protein consisting of MBP and orfD, perhaps because of the instability of the fusion protein. While we were able to obtain fusion proteins for MBP-orfA, MBP-orfB, MBP-orfC, and MBP-orfH and were able to obtain antibodies to the fusion proteins, we did not detect the predicted proteins in extracts of *B. pertussis*. A variety of explanations may account for these findings, including the possibility that the fusion proteins induce very little antibody that recognizes the Ptl proteins or that Ptl proteins are present in minute quantities in the cell and are therefore difficult to detect.

**Localization of PtlE, PtlF, and PtlG in *B. pertussis*.** *B. pertussis* cells were fractionated into soluble and particulate fractions representative of the cytosolic or periplasmic compartments and membranes, respectively. As shown in Fig. 5, PtlE, PtlF, and PtlG were found in the particulate fraction.

Differential solubilization of bacterial membrane proteins in Triton X-100 has been used as an indicator of whether a protein is located in the inner or the outer membrane, since Triton X-100 is believed to preferentially solubilize inner membrane proteins of *B. pertussis* (3, 7, 26). Other methods such as isopycnic centrifugation do not separate *B. pertussis* membranes into distinguishable fractions corresponding to inner and outer membranes (7) and therefore would not be useful in this regard. The total membrane fraction was treated with 2% Triton X-100, and the detergent soluble proteins were separated from insoluble proteins by centrifugation. PtlF and PtlG were insoluble in Triton X-100 (Fig. 6). PtlE was partially soluble in the detergent.
Examination of related *B. pertussis* strains and other *Bordetella* spp. for the production of Ptl proteins. *B. pertussis* BP356 contains a Tn5 insertion within the gene coding for subunit S3 of PT (34). Others have reported that this strain made PT subunits S1, S2, and S4, and S5, but the subunits were not secreted, and they concluded, therefore, that S3 was necessary for secretion of PT (19, 22). Since the Ptl genes are located directly downstream from the Tn5 element, we wanted to determine whether the transposon affected production of Ptl proteins in this strain. Using antibodies which recognize PtlE and PtlF, we probed this strain for the presence of these Ptl proteins. BP356 did not contain detectable amounts of PtlF (Fig. 7) or PtlE (data not shown), although this strain produced normal amounts of FHA, another bvg-regulated protein. FHA is readily degraded and therefore appears as one major band as well as several breakdown products. BP356 also contains detectable levels of the S1 subunit of PT (Fig. 7).

Other *Bordetella* spp. have been reported to contain regions homologous to the *ptl* genes (2). Extracts of *B. parapertussis, B. bronchiseptica*, and *B. pertussis* 18323, a pertussis strain which has been reported to be an intermediate in the evolution from *B. pertussis* to *B. parapertussis* and *B. bronchiseptica* (1), were probed for protein which binds to antibodies which recognize PtlF. While *B. pertussis* 18323 produced PtlF, *B. parapertussis* 9305 and *B. bronchiseptica* Bb55 did not produce a protein that reacted with PtlF antibodies (Fig. 8). A few faint bands having apparent molecular weights different from that of PtlF were observed in extracts from *B. bronchiseptica*; however, all of these bands were also present in extracts from the modulated form of the organism. Also, while *B. pertussis* 18323 produced PtlE, extracts from *B. parapertussis* 9305 and *B. bronchiseptica* Bb55 did not contain a protein that reacted with antibodies that recognize PtlE (data not shown).

**DISCUSSION**

The existence of the Ptl proteins is predicted from nucleotide sequence analysis of a region of the *B. pertussis* chromosome which is necessary for efficient secretion of PT from the bacterium (6, 37). Using antibodies raised to fusion proteins containing the predicted primary sequence of PtlE, PtlF, and PtlG, we have detected these Ptl proteins in extracts of *B. pertussis*; this constitutes the first identification of any of the Ptl proteins.

PtIE has an apparent molecular weight on SDS-polyacrylamide gels of 30,000, which is in good agreement with a molecular weight of 31,010 predicted from the DNA sequence (37). PtIE cofractionates with membranes of *B. pertussis* extracts. This protein is partially soluble in Triton X-100, suggesting that PtIE has an inner membrane location but might have some association with components of the outer membrane. An inner membrane location for this protein is consistent with its amino acid sequence, which predicts that the protein has no signal sequence which would target the protein to the outer membrane. PtIE exhibits 32% identity with the VirB8 protein of *A. tumefaciens* over a stretch of 180 amino acids (37). VirB8 has been reported to be an inner membrane protein that is likely associated with outer membrane components (28, 29).

PtIF has an apparent molecular weight on SDS-polyacrylamide gels of 31,000. The predicted molecular weight of the protein encoded by the entire orfF is 29,471 (37); however, the predicted protein has an N-terminal sequence, MMAARM MAAGLAATALSAHA, which has the characteristics of a signal sequence (5). The mature form of PtIF would therefore be predicted to have a molecular weight of 27,541. PtIF cofractionates with the membranes of *B. pertussis* extracts and is insoluble in Triton X-100, suggesting that it is an outer membrane protein or associated with components of the outer membrane. PtIF exhibits 27% identity with VirB9 of *A. tumefaciens* over a stretch of 252 amino acids (37). VirB9 also has a signal sequence and is found in both inner and outer membrane fractions (28). Others have postulated that VirB9 is actually an inner membrane protein that is associated with components of the outer membrane, causing it to fractionate in both inner and outer membrane fractions (28).

PtG, which migrates with an apparent molecular weight of 38,000, exhibits 34% identity with VirB10 over a 270-amino-acid stretch (37). PtG cofractionates with the membranes of *B. pertussis* extracts and is insoluble in Triton X-100. VirB10 was found in the inner membrane fraction of *A. tumefaciens* (33) but is thought to be tightly associated with outer membrane components, causing it to fractionate with the outer membrane if certain fractionation methods, including differential solubility in Sarkosyl, are used (28). Since PtG has no obvious signal
sequence, it may also be an inner membrane protein that
associates tightly with components of the outer membrane.

We have used antibodies to Ptl proteins to examine a strain
of B. pertussis which has previously been shown to be defective
in the secretion of PT subunits (19, 22). We found that BP356,
which contains a Tn5 insertion in the structural gene for
subunit S3 of PT (19, 22), does not produce detectable levels of
PTIE or PtlF. Thus, the presence of the Tn5 insertion in the S3
gene appears to affect expression of at least certain of the ptl
genes located directly downstream of the insertion, in addition
to its effect on production of the S3 subunit that has been
reported (19, 22). Previously, others concluded that S3 is likely
essential for export of PT, since other PT subunits are pro-
duced by BP356 but they are not secreted (19, 22). However,
this conclusion may need to be reexamined given our finding
that production of at least certain of the Ptl proteins is also
affected by the transposon. Further investigation is needed
to determine why the transposon affects expression of the ptl
genes, since it has been suggested that ptl genes which encode
the PT subunits and ptl genes are parts of different operons
(37).

The Bordetella genus contains four species, B. pertussis, B.
parapertussis, B. bronchiseptica, and Bordetella avium. While B.
pertussis is the only one of these species that produces PT (2),
all strains of B. parapertussis and the majority of B. bronchisept-
tica strains contain the ptl genes (2, 20). In addition to the ptl
genes, these closely related species also contain regions homol-
ogous to the entire ptl region (2). Arico and Rappuoli (2)
sequenced the ptl region and portions of the ptl region
(including pIAl, pIIB, and part of pIC) from B. parapertussis and
B. bronchiseptica and found that these regions have 98.6 and
96% identity, respectively, to the ptl-ptime regions of B. pertussis.
At the amino acid level, the DNA sequence predicts that these
Ptl proteins from B. parapertussis and B. bronchiseptica would
have an average of 99 and 95% identity, respectively, to the
predicted amino acid sequence of the corresponding Ptl pro-
teins from B. pertussis. Using antibodies that recognize the B.
pertussis Ptl proteins, we examined B. parapertussis and B.
bronchiseptica extracts for the presence of PTIE and PtlF.

Neither species produced detectable levels of these proteins.
While it is possible that the lack of detection of these Ptl
proteins in B. parapertussis and B. bronchiseptica is due to lack
of cross-reactivity of the antibodies, this explanation seems
unlikely given the high level of homology that has been
described between the ptl-ptime genes of Bordetella spp. (2).
The B. parapertussis strain that we used in our study (strain 9305)
is known to contain the entire ptl region (2). Therefore, while the
ptl genes are present, they are likely not expressed, a finding
similar to that observed for the ptl genes of B. parapertussis and
B. bronchiseptica.

In contrast to B. parapertussis and B. bronchiseptica strains,
B. pertussis 18323, a pertussis strain that has been described as
an intermediate in the evolution of B. parapertussis and B.
bronchiseptica from B. pertussis (1), does produce detectable
levels of PTIE and PtlF. Since B. pertussis 18323 produces PT
(1), this organism would need the Ptl proteins for secretion of
the toxin. In contrast, B. parapertussis and B. bronchiseptica do
not produce PT (2) and therefore would not have a need for
the Ptl proteins if these transport proteins were used exclu-
sively for secretion of PT and not for export of other proteins
common to the Bordetella spp.

Previously, others introduced the ptl genes from B. pertussis
into strains of B. parapertussis and B. bronchiseptica, including
B. bronchiseptica Bb55, which we have used in this study, in an
attempt to produce PT in other Bordetella spp. They attempted
to produce PT in other bordetellae, since B. pertussis is a slowly
growing organism that is not ideal for production of the
amounts of PT needed in the manufacture of vaccine compo-
nents for acellular pertussis vaccines (16, 30). However, their
results indicated that while PT can be produced in these
organisms, the toxin is not secreted and would therefore be
difficult to purify. Our lack of ability to detect PTIE and PtlF in
B. parapertussis and B. bronchiseptica suggests that, at a
minimum, both the ptx and ptl genes from B. pertussis need to
be introduced into these Bordetella spp. before secretion of PT
could be achieved.

In this report, we have shown that PTIE, PtlF, and PtlG can be
detected in B. pertussis but not in other Bordetella spp. All
three Ptl proteins appear to be localized in the bacterial
membrane. A more precise localization within the bacterial
envelope (i.e., inner versus outer membrane) is difficult to
make because of technical problems associated with separation
of the inner and outer membranes of B. pertussis. The
membrane localization of Ptl proteins suggests that these proteins
might form a gate or channel used in the transport process.
The VirB proteins of A. tumefaciens have been postulated to for
a channel that spans both the inner and the outer membranes
of the bacterium, which may open and close in response to the
transportable substrate (28). Transport of the T-DNA has been proposed to occur by a one-step mechanism in which the T complex crosses the membrane barriers through
a channel that spans both membranes. PT might be trans-
ported across the two bacterial membranes by a single-step
mechanism analogous to that proposed for T-DNA. Alterna-
tively, transport of PT might occur by a two-step mechanism in
which the individual subunits are first transported across the
inner membrane, perhaps in an unfolded or partially folded
state. The molecule might then assemble in the periplasmic
space and would then be transported via the Ptl system across
the final membrane barrier. Such a two-step process has been
proposed for secretion of pullulanase from E. coli (24) and
secretion of enterotoxin from Vibrio cholerae (8).

REFERENCES
Bordetella bronchiseptica contain transcriptionally silent pertussis
envelope proteins of Bordetella pertussis. Infect. Immun. 54:109–
117.
quantitation of microgram quantities of protein utilizing the
of protein secretion: the role of the signal sequence. Adv. Protein
accessory genes located downstream from the pertussis toxin
1981. Phase shift markers in Bordetella: alterations in envelope
subunits into the periplasm occurs during their secretion from
antigenic modulation of Bordetella pertussis in modified Horni-
eukaryotic cells: binding, entry, and activation. FASEB J. 6:2684–
2690.


