Requirements for Assembly of PtlH with the Pertussis Toxin Transporter Apparatus of *Bordetella pertussis*

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PtlH is an essential component of the Ptl system, the type IV transporter responsible for secretion of pertussis toxin (PT) across the outer membrane of *Bordetella pertussis*. The nine Ptl proteins are believed to interact to form a membrane-spanning apparatus through which the toxin is secreted. In this study, we monitored the subcellular localization of PtlH in strains of *B. pertussis* lacking PT, lacking other Ptl proteins, or from which ATP has been depleted in order to gain insight into the requirements for assembly of PtlH with the remainder of the Ptl transporter complex that is thought to be tightly embedded in the membrane. We found that PtlH is exclusively localized to the inner membrane fraction of the cell in a wild-type strain of *B. pertussis*. In contrast, PtlH localized to both the cytoplasmic and inner membrane fractions of a mutant strain of *B. pertussis* that does not produce PT. In comparison to how it localized in wild-type strains of *B. pertussis*, PtlH exhibited aberrant localization in strains lacking PtlD, PtlE, PtlF, and PtlG. We also found that localization of PtlH was perturbed in *B. pertussis* strains that were treated with carbonyl cyanide m-chlorophenylhydrazine and sodium arsenate, which are capable of depleting cellular ATP levels, and in strains of *B. pertussis* that produce an altered form of PtlH that lacks ATPase activity. When taken together, these results indicate that tight association of PtlH with the membrane, likely through interactions with components of the transporter-PT complex, requires the toxin substrate, a specific subset of the Ptl proteins, and ATP. Based on these data, a model for the assembly of the Ptl transporter-PT complex is presented.

Pertussis toxin (PT) is an essential virulence factor that is secreted by *Bordetella pertussis* (40), the causative agent of pertussis (whooping cough). PT is a large, complex, oligomeric protein that interferes with signaling pathways in host cells by ADP-ribosylating a family of GTP-binding regulatory proteins (16, 18). The toxin, which belongs to the AB class of bacterial toxins, is composed of five different subunits, S1, S2, S3, S4, and S5, found in a ratio of 1:1:1:2:1 (24, 25, 37). The enzymatically active S1 subunit (the A component) sits atop a ring formed by the remaining subunits that make up the binding component, or the B oligomer, of the toxin (33).

Secretion of PT from the bacterium requires a transport system that comprises nine accessory proteins known as the Ptl proteins (41). The genes encoding the transport system are located directly downstream from the genes encoding the toxin structural subunits (Fig. 1A). The Ptl transport apparatus is a member of the type IV family of secretion systems that are involved in the transport of proteins and/or DNA from bacterial cells (8, 42). The Ptl system displays considerable homology to other type IV transporters, including the prototypic type IV transporter, the VirB/D4 system of *Agrobacterium tumefaciens*, which has been studied extensively. Because of the similarities between these systems, much can be surmised concerning the general architecture of the Ptl transporter based on what is known about the VirB/D4 system. However, extrapolations from the VirB/D4 system to the Ptl system have certain limitations because distinct differences exist between the Ptl and VirB/VirD4 systems. In particular, these two systems differ dramatically in regards to their substrates and the locations within the bacteria at which the substrates are believed to initially interact with the transporters. The substrates for the VirB/D4 transporter are cytoplasmic proteins, certain of which are covalently bound to DNA. These substrates are thought to interact with the VirB/D4 transporter at the cytoplasmic surface of the inner membrane through initial interaction with VirD4, a protein that is lacking in the Ptl system (2, 7). In contrast, the substrate for the Ptl system, PT, is an oligomeric protein that is transported via a Sec-like system to the bacterial periplasm independent of the Ptl transporter (41). Thus, PT is believed to interact initially with the Ptl transporter in the periplasm rather than the cytoplasm and therefore must interact with the Ptl transporter in a manner distinctly different from the manner in which the substrates of the VirB/D4 transporter initially interact with that assembly. Currently, little is known about how and when PT initially interacts with its transporter.

In our previous studies, we examined mutants of *B. pertussis* that lack specific Ptl proteins or the toxin itself in order to gain a better understanding of the transporter and its interaction with PT. In the course of these studies, we noted that subcellular localization of PtlH, a critical component of the Ptl transporter, is altered in certain of these mutants. PtlH is a member of a family of type IV transporter proteins that possess ATPase activity and which have been demonstrated to be peripheral inner membrane proteins (9, 27). Because tight association of peripheral membrane proteins with the membrane can depend on their interaction with other integral membrane proteins (19, 29), we monitored the subcellular localization of PtlH in specific mutant strains of *B. pertussis* in order to gain insight into
the requirements for assembly of PtlH with the remainder of the membrane-embedded Ptl transporter. In this study, we examined the subcellular localization of PtlH in mutants of B. pertussis lacking PT and/or specific Ptl proteins. We also examined localization of PtlH in the absence of ATP binding. We found that tight association of PtlH with the membrane is dependent on the toxin substrate, certain other Ptl proteins, and ATP binding and/or hydrolysis, information that sheds light on the requirements for transporter assembly, interactions of PT with the transporter, and the sequence of events that take place during secretion of PT from B. pertussis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains of B. pertussis and Escherichia coli as well as the plasmids used in this study are listed in Table 1. B. pertussis strains were grown at 37°C on Bordet-Gengou (BG) agar.

Construction of B. pertussis in-frame deletion mutants. B. pertussis in-frame chromosomal deletion mutants were constructed by homologous recombination as previously described (14, 35). Briefly, approximately 900-bp regions flanking upstream and downstream of the desired gene to be deleted were amplified using primers mapping in upstream and downstream regions, respectively. Amplified products were combined either by overlapping PCR or by ligation and cloned in E. coli, was grown on BG agar plates. Cells were suspended in PBS to an A600 of 2.0. Cells (6 ml of suspended culture) were collected by centrifugation at 10,000 × g, suspended in 1.25 ml lysis buffer (10 mM Tris-HCl, pH 8.0, 1 M sucrose, 0.5% Zwittergent-316, and 0.1 mg of lysozyme per ml) and subjected to ultracentrifugation at 144,000 × g for 1 h. After ultracentrifugation, the pellet (total membrane fraction) and the supernatant (periplasmic/cytoplasmic fraction) were treated with 1.5% Sarkosyl overnight at 4°C on a rotator. Detergent solubilized fractions were subjected to ultracentrifugation at 144,000 × g for 1 h. Proteins were precipitated from detergent-solubilized fractions (100 μl) by ethanol, suspended in SDS-PAGE loading buffer, and subjected to SDS-PAGE according to procedure of Laemmli (21), followed by transfer to nitrocellulose and immunoblot analysis using antisera raised against His6-PtlH at dilution of 1:2,000. Blots were developed using the ECL system (Amersham). Densitometry was performed using AlphaEaseFC software (Alpha Innotech Corp. USA).

Preparation of cell extracts and fractionation. (i) Separation of soluble and total membrane fractions. B. pertussis strains were grown on BG agar plates and suspended in phosphate-buffered saline (PBS) to an A600 of 2.0. Cells (6 ml of suspended culture) were collected by centrifugation at 10,000 × g, and resuspended in 1 ml of 0.2 M Tris-HCl, pH 8.0. Equivalent portions of the Triton X-100 fractions were precipitated by ethanol and then subjected to immunoblot analysis.

Preparation of cell extracts and fractionation. (ii) Triton X-100 solubilization of the total membrane fraction. The membrane fraction, prepared as described above, was suspended in 10 mM Tris-HCl (pH 8.0) containing 2% Triton X-100. The preparation was incubated overnight at 4°C and then centrifuged at 144,000 × g for 1 h. The pellet (Triton X-100- insoluble fraction) and the supernatant (periplasmic/cytoplasmic fraction) were treated with 1.5% Sarkosyl overnight at 4°C on a rotator. Detergent solubilized fractions were subjected to ultracentrifugation at 144,000 × g for 1 h. Proteins were precipitated from detergent-solubilized fractions (100 μl) by ethanol, suspended in SDS-PAGE loading buffer, and subjected to SDS-PAGE according to procedure of Laemmli (21), followed by transfer to nitrocellulose and immunoblot analysis using antisera raised against His6-PtlH at dilution of 1:2,000. Blots were developed using the ECL system (Amersham). Densitometry was performed using AlphaEaseFC software (Alpha Innotech Corp. USA).

Preparation of cell extracts and fractionation. (iii) Isolation of periplasmic and cytoplasmic fractions. BPS36ΔptlH(pTH22), which contains a plasmid capable of expressing the phoA gene of E. coli, was grown on BG agar plates. Cells were suspended in PBS to an A600 of 2.0, collected by centrifugation at 10,000 × g, and resuspended in 1 ml of 0.2 M Tris-HCl, pH 8.0. An equal volume of 0.2 M Tris-HCl, pH 8.0, containing 1 M sucrose, 0.5% Zwittergent-316, (Calbiochem, La Jolla, CA), and 0.1 mg of lysozyme per ml was added. Cells were then exposed to mild osmotic shock by the addition of 4 ml of water to release the periplasmic contents of the cell as previously described (32). After a shaking for 2 h at room temperature, the cell suspension was centrifuged for 30 min at 8,000 × g and the supernatant (cytoplasmic fraction) was collected. Cytoplasmic and periplasmic supernatants were subjected to ultracentrifugation at 144,000 × g for 1 h to remove any membrane debris. After ultracentrifugation, aliquots of the supernatants were used to measure alkaline phosphatase activity as described by Brickman and Beckwith (4). Immunoblot analysis, periplasmic and cytoplasmic fractions were treated with 1.5% Sarkosyl overnight at 4°C on a rotator. Detergent solubilized fractions were again subjected to ultracentrifugation at 144,000 × g for 1 h. Proteins were precipitated from detergent-solubilized fractions (100 μl) by ethanol and subjected to immunoblot analysis as described above.
TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>lacZAM15Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK- mK-) thi-1 gyrA96 relA1</td>
<td>Gibco-BRL</td>
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<tr>
<td>SM10pir</td>
<td>thi-1 pir-1 lacY1 supE44 recA4::RP4-2-Tc::Mu lpirR6K</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BL-21(DE3)</td>
<td>F- ompT pRPLV (lon) hsdS2 (rK- mK-)</td>
<td></td>
</tr>
<tr>
<td><strong>B. pertussis strains</strong></td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>BP536</td>
<td>Wild-type, nalidixic acid-resistant, streptomycin-resistant derivative of Tohama I</td>
<td></td>
</tr>
<tr>
<td>BP36Δptl(5073-3683)</td>
<td>BP536 with an in-frame deletion in the ptx region, from nucleotide 507 to nucleotide 3683</td>
<td>This study</td>
</tr>
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<td>BP36Δptl</td>
<td>BP536 with a 7.4-kb in-frame deletion in the ptx-rtl region, from nucleotide 3626 to nucleotide 11039</td>
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<td>BP36Δptlptl</td>
<td>BP536 with an 11.5-kb in-frame deletion in ptx-rtl genes, from nucleotide 425 to nucleotide 11971</td>
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<td>BP36ΔptlII</td>
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<td>BP536 with an in-frame deletion in the ptx-rtl region, from nucleotide 9974 to nucleotide 10972</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pUFRO47</td>
<td>Broad-host-range (IncW) vector, Mob, lacZα, gentamicin resistant</td>
<td>Promega</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>AmpC-resistant cloning vector</td>
<td></td>
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<tr>
<td>pS11129</td>
<td>Gen+, Amp+, Smα allelic exchange vector</td>
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</tr>
<tr>
<td>pQE80</td>
<td>Amp′, cis-repressed, high-level expression vector of N-terminally Hisα-tagged proteins</td>
<td>QIAGEN</td>
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<td>pTH18</td>
<td>pUFRO47 containing ptsS2, ptsS4, ptsS5, and ptsS3 under lacZ promoter control</td>
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<td>pTH19</td>
<td>pRK415 containing the ptx-rtl promoter region and ptsI</td>
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<td>pAV9</td>
<td>pGEXκp-1 containing ptlH (nucleotides 10973 to 11992) at the 3′ end of the gene encoding GST</td>
<td>This study</td>
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<tr>
<td>pAV21</td>
<td>pGEXκp-1 containing ptlH(K176A) at the 3′ end of the gene encoding GST</td>
<td>This study</td>
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<tr>
<td>pAV31</td>
<td>pUFRO47 containing ptlH under ptx-rtl promoter control</td>
<td>This study</td>
</tr>
<tr>
<td>pAV32</td>
<td>pUFRO47 containing ptlH(K176A) under ptx-rtl promoter control</td>
<td>This study</td>
</tr>
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<td>pAV69</td>
<td>pQE80 containing ptlH with a Hisα tag at the 5′ end of the gene</td>
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<tr>
<td>pTH22</td>
<td>pUFRO47 containing the phoA gene of E. coli under lacZ promoter control</td>
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Depletion of ATP from B. pertussis. BP536 strain was inoculated in Stainer-Scholte medium at an initial A600 of 0.25 to 0.30 and grown to an A600 of 1.1 to 1.5 for approximately 40 h. After 40 h of culture growth, cells were treated with 50 μM of carbonyl cyanide m-chlorophenylhydrazine (CCCP) or 50 mM of sodium arsenate for 30 min with shaking. After treatment, cells were harvested, centrifuged, and suspended in an amount of PBS equal to the total of the harvested culture volume. Cellular ATP levels were measured in untreated or CCCP- or sodium arsenate-treated cells by using an ATP bioluminescence assay kit (Promega) as per the manufacturer’s instructions. Untreated and treated cells were also subjected to ultracentrifugation for separation of soluble and membrane fractions, treated with 1.5% Sarkosyl, and subjected to immunoblot analysis as described above.

Cloning, expression, and purification of GST-PtlH in E. coli. A 1,019-bp fragment of the ptx-rtl operon extending from nucleotide 10973 through nucleotide 11992, corresponding to ptlH, was amplified using primers (5′ GGAAATT CGTGAATGATGCCGCGCCGGATCG 3′ [with its EcoRI site underlined]) and (5′ CCCGGGTCGGATCCTAGGGCCGGGAGGTGATT 3′ [with its Xhol site underlined]) and genomic DNA isolated from the B. pertussis Tohama I strain. The nucleotide numbering system used for the ptx-rtl operon has been previously described (25, 41). The amplified product was cloned into the pGEM-T Easy plasmid (Promega) and sequenced. For expression, the ptlH gene was further subcloned in frame into the EcoRI and Xhol sites at the 3′ end of the gene encoding glutathione S-transferase (GST) of pGEXκp-1 (Amerham). The resulting construct, pAV9, was transferred to E. coli BL-21 for expression and purification. The following procedure was used to purify the GST fusion protein from pAV9. Briefly, cells were inoculated into LB medium containing 100 μg/ml ampicillin and allowed to grow to an A600 of 0.6 at 30°C. Protein expression was reduced glutathione. Similar methods were used to purify a mutant form of GST-PtlH expressed in pAV21.

ATPase activity assays. ATPase activity of GST-PtlH and GST-PtlH(K176A) fusion protein was subjected to electrophoresis on native 4 to 20% acrylamide gels in 25 mM Tris-MnCl2 gel with 50 mM MnCl2 with 0.1% Triton X-100. GST fusion proteins were eluted from glutathione Sepharose beads using 10 mM of reduced glutathione. Similar methods were used to purify a mutant form of GST-PtlH expressed in pAV21.

PCR mutagenesis in Walker A box motif of PtlH. A point mutation was made in the coding sequence of ptlH to change the conserved lysine at amino acid position 176 of PtlH to alanine by using the QuikChange II site-directed mutagenesis kit (Stratagene). Primers mapping at the mutagenesis site (5′ CGGG CCAAGACCGGTTCGGGCGGCACCACTTAGTGAAGCGCTTGA GGGCC 3′ and 5′ CGGGTACAAGCGGCTATCATAATGTTGCGCCGGGAA CCGGCTCGGCGCGG 3′ [underlining indicates the mutagenesis site]) were used for the amplification. After mutagenesis, plasmids were sequenced for the desired mutations and to ensure that no other mutation was introduced in the ptlH gene. Mutated ptlH, designated ptlH(K176A), was further subcloned in frame [Calbiochem], 1 mM phenylmethylsulfonyl fluoride). Cells were lysed by sonication and bacterial debris was pelleted at 10,000 × g for 20 min. The clear supernatant was added to washed glutathione Sepharose 4B beads (Amersham) and mixed gently at 4°C overnight. The beads were centrifuged at 500 × g for 5 min and washed several times with PBS containing 0.1% Triton X-100. GST fusion proteins were eluted from glutathione Sepharose beads using 10 mM of reduced glutathione. Similar methods were used to purify a mutant form of GST-PtlH expressed in pAV21.
Subcellular localization of PtlH in a wild-type strain of *B. pertussis*. In order to localize PtlH in *B. pertussis*, we fractionated bacterial cells into soluble (cytoplasmic and periplasmic) and total (inner and outer) membrane fractions. Visualization of PtlH on immunoblots by using polyclonal anti-PtlH antiserum required treatment of the fractions with 1.5% Sarkosyl. Sarkosyl treatment most likely disrupts higher-molecular-weight complexes of PtlH, such as oligomeric forms of PtlH or PtlH complexes with other proteins, thus allowing PtlH to enter the gel and migrate as a monomeric species.

When we examined the wild-type strain of *B. pertussis*, BP536, we found that PtlH migrated as a protein of approximately 37 kDa that was detected exclusively in the membrane fraction of the cells (Fig. 2A, lanes 1 and 2). Immunoblot analysis of a strain of *B. pertussis* containing an in-frame deletion of PtlH, BP536ΔptlH, showed no reactivity in either the membrane or soluble fractions (Fig. 2B, lane 1, Triton X-100-insoluble fraction). Positions of molecular size markers (in kilodaltons) are indicated at the left. The arrow indicates the protein band corresponding to PtlH.

Because our results indicated that PT is required for localization of PtlH exclusively to the membrane, we next determined whether either the enzymatically active S1 subunit or the B oligomer (subunits S2, S3, S4, and S5) might be sufficient to direct localization of PtlH exclusively to the membrane. We introduced plasmids expressing ptxS1 (ptxH19) or ptxS2-pxtS5 (ptxH18) into BP536ΔptxS (507-3683). In both cases, we observed that approximately 35 to 50% of the cellular PtlH partitioned to the soluble fraction (Fig. 3A, lanes 5 to 8) and that the localization of PtlH in these strains did not differ significantly from that of BP536ΔptxS (507-3683) (Fig. 3B). However, when the enzymatically active S1 subunit and B oligomer were produced simultaneously in BP536ΔptxS (507-3683) by introducing both pTH19 and pTH18, we observed that PtlH localized exclusively to the membrane fraction (Fig. 3A, lanes 9 and 10), indicating that an intact PT molecule is necessary for tight association of PtlH with the membrane.

In the experiment whose data are shown in Fig. 3, the soluble fraction consisted of both the cytoplasmic and periplasmic contents of the cell. In order to further localize the soluble form of PtlH, we separated the cytoplasmic and periplasmic contents of BP536ΔptxS (507-3683)(pTH12). This strain expresses a gene encoding alkaline phosphatase, which can be used as a marker for the periplasmic contents of the cell (12). After separating cytoplasmic and periplasmic fractions of the cell by using the Zwittergent-lysozyme procedure described in Materials and Methods, we found that approximately 90% of the alkaline phosphatase was found in the periplasmic fraction, with only about 10% contaminating the cytoplasmic fraction (Fig. 4A), indicating that the method satisfactorily removes the periplasmic contents of the cell from its cytoplasmic contents. We then examined both the periplasmic and cytoplasmic fractions for PtlH content. As shown in Fig. 4B, PtlH partitioned exclusively to the cytoplasmic fraction, indicating that the soluble form of PtlH is located in the cytoplasm.

**Localization of PtlH in strains lacking other Ptl proteins.** We next asked whether the presence of the other Ptl proteins influences localization of PtlH. BP536Δptl, which does not produce the Ptl proteins but does produce PT, was complemented in trans with a plasmid that expresses wild-type *ptlH* (pAV31). As a control, the wild-type strain was also complemented with this plasmid. Immunoblot analysis revealed that PtlH partitioned exclusively to the membrane fraction of the wild-type strain into which pAV31 had been introduced (Fig. 5, lanes 3 and 4). Approximately 50% of the total cellular PtlH could be seen in the soluble fraction of BP536Δptl(pAV31), a
strain of \textit{B. pertussis} lacking all Ptl proteins except PtlH, which was provided in \textit{trans} (Fig. 5, lanes 7 and 8). These observations suggest that the presence of other Ptl proteins is critical for tight association of PtlH with the membrane.

Because both PT and the Ptl proteins are required for wild-type localization of PtlH, we asked whether the subcellular location of PtlH would be more perturbed in a strain lacking both of these components than in a strain lacking only the Ptl proteins. When we examined the cellular localization of PtlH in BP536/\textit{H9004} \textit{ptl} (pAV31), a strain lacking all \textit{ptl} genes except \textit{ptlH}, which was provided in \textit{trans}, we found that PtlH partitioned in a manner similar to that of BP536/\textit{H9004} \textit{ptl} (pAV31) (Fig. 5, compare lanes 5 and 6 to lanes 7 and 8). The finding that about half of the PtlH of the cell associates with the membrane even in the absence of other members of the Ptl transporter-PT complex may be due to an intrinsic affinity of PtlH for the membrane. Other homologs of PtlH, in particular, VirB11, are found to be associated with the inner membrane of the bacterium, even in the absence of other transporter proteins (27).

In order to gain insight into which Ptl protein(s) might be necessary for tight association of PtlH with the membrane, we examined the localization of PtlH in strains of \textit{B. pertussis} which contained an in-frame deletion of \textit{ptlA}, \textit{ptlD}, \textit{ptlE}, \textit{ptlF},
PtlH mutant (approximately 30 to 50% of total cellular PtlH was found in the soluble fraction of the mutant, and approximately 30 to 50% of total cellular PtlH was found in the soluble fraction of the wild-type strain. Thus, as expected, the in-frame ptlA, ptlE, ptlF, ptlG, and ptlI mutations do not appear to affect expression of the downstream ptlH gene. The decrease in the total amount of PtlH observed in the strain lacking PtlD is likely due to stabilization of PtlH by PtlD since complementation of the mutant with ptlD can restore PtlH levels to those of the wild-type strain (A. Cheung and D. Burns, unpublished data). Localization of PtlH in cells from which ATP has been depleted and localization of altered forms of PtlH lacking ATPase activity. PtlH is homologous to transfer ATPases that can exhibit ATP-dependent alterations in conformation (30). Others have suggested that these conformational changes might affect membrane binding and interaction with other components of the transport apparatus (9, 30). We therefore examined the localization of PtlH in bacteria from which ATP has been depleted or of mutant forms of PtlH that exhibit altered ATPase activity in order to assess the dependence of PtlH localization on ATP. Previously, PtlH was classified as an ATPase, based on homology with other members of this family of transfer ATPases. PtlH contains the amino acid sequence motifs characteristic of this family of proteins that are thought to be critical for ATPase activity (Fig. 1B). In this study, we confirmed the ATPase activity of PtlH by examining the ability of a GST-PtlH fusion that had been produced in E. coli to hydrolyze ATP. Affinity-purified GST-PtlH fusion protein, along with a negative control protein, affinity-purified GST-PtlI, was subjected to nondenaturing PAGE. The gel was then stained to assess the formation of P, formed after incubation with ATP. The purified protein band corresponding to GST-PtlH exhibited ATPase activity (Fig. 7B, lane 1), whereas the negative control protein did not (Fig. 7B, lane 3). Mutation of the highly conserved alanine residue (mutated from lysine) at amino acid position 176 located in the Walker A box motif of PtlH (Fig. 1B) resulted in loss of this ATPase activity (Fig. 7B, lane 2) as expected since the Walker A box motif of nucleotide-binding proteins has been shown to interact directly with nucleotides and to be essential for the NTP-dependent functions of these proteins (27, 34, 38). In order to assess whether ATP affects the localization of PtlH, we treated B. pertussis cells with either CCCP, which collapses the transmembrane potential and dissipates cellular ATP, or sodium arsenate, which lowers ATP levels without the exception of the ptiD mutant, was similar to that of the wild-type strain. Thus, as expected, the in-frame ptiA, ptiE, ptiF, ptiG, and ptiI mutations do not appear to affect expression of the downstream ptiH gene. The decrease in the total amount of PtlH observed in the strain lacking PtlD is likely due to stabilization of PtlH by PtlD since complementation of the mutant with ptiD can restore PtlH levels to those of the wild-type strain (A. Cheung and D. Burns, unpublished data).

Localization of PtlH in strains lacking other Ptl proteins. A plasmid containing ptiH (pAV31) was introduced into BP536 (wild-type B. pertussis), BP536Δptxptl (lacking PT and Ptl proteins), or BP536Δptl (lacking Ptl proteins). Soluble (S; 100 μl) and membrane (M; 100 μl) fractions of these strains were prepared as described in Materials and Methods and subjected to immunoblot analysis using polyclonal antibodies specific for PtlH. Lanes 1 and 2, BP536; lanes 3 and 4, BP536(pAV31); lanes 5 and 6, BP536Δptxptl(pAV31); lanes 7 and 8, BP536Δptl(pAV31). Positions of molecular size markers (in kilodaltons) are indicated at the left. The arrow indicates the protein band corresponding to PtlH. Results are representative of two independent experiments.
affecting the transmembrane potential (6, 23). After treatment of the bacteria with either CCCP or sodium arsenate, ATP levels were measured to be 2% of the level in untreated cells. Treatment of cells with either energy poison perturbed the localization of PtlH. As can be seen in Fig. 8A, when cells were exposed to either CCCP or sodium arsenate, the portion of PtlH that partitioned to the soluble fraction was larger than that for untreated cells.

We also examined whether PtlH(K176A), which lacks ATPase activity, localized in a manner distinct from that of the native form of PtlH. In order to accomplish this, we introduced a plasmid (pAV32) capable of expressing \textit{ptlH}^{(K176A)} into \textit{BP536}/\textit{H9004 ptlH} and examined localization of PtlH(K176A) in this strain. For a control, we also introduced a plasmid (pAV31) capable of expressing wild-type \textit{ptlH} into \textit{BP536ΔptlH}. \textit{BP536ΔptlH(pAV32)} exhibited partial distribution of PtlH(K176A) in the soluble fraction as well as the membrane fraction (Fig. 8B, lanes 3 and 4), whereas native PtlH localized strictly to the membrane fraction in \textit{BP536ΔptlH(pAV31)} (Fig. 8, lanes 1 and 2). We have previously shown that production of PtlH(K176A) in a wild-type background of \textit{B. pertussis} results in a dominant negative phenotype (20) suggesting that this form of the protein can properly fold and interact with other proteins.

**DISCUSSION**

In this study, we found that localization of PtlH in the \textit{B. pertussis} bacterium is dependent both on Ptl proteins and on the toxin substrate. Our finding that both the toxin and other Ptl proteins are required for proper localization of PtlH suggests that PtlH may interact either directly or indirectly with one or more of these proteins. Given the homologies that exist between the Ptl system and other type IV transporters, it is expected that PtlH would interact directly with other Ptl transporter proteins (9), possibly PtlF, since data from yeast two-hybrid studies of the VirB proteins have demonstrated a direct interaction between VirB11 (PtlH homolog) and VirB9 (PtlF homolog) (39). While direct interactions of PtlH with other Ptl proteins would be predicted, our finding that PT is required for proper localization of PtlH was unexpected and provides im-

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**FIG. 6.** Localization of PtlH in \textit{BP536} strains lacking different Ptl proteins. Soluble (S; 100 μl) and membrane (M; 100 μl) fractions of \textit{BP536} strains lacking specific Ptl proteins were prepared as described in Materials and Methods and subjected to immunoblot analysis using polyclonal antibodies specific for PtlH. (A) Lanes 1 and 2, \textit{BP536}; lanes 3 and 4, \textit{BP536ΔptlA}; lanes 5 and 6, \textit{BP536ΔptlD}. (B) Lanes 1 and 2, \textit{BP536}; lanes 3 and 4, \textit{BP536ΔptlE}; lanes 5 and 6, \textit{BP536ΔptlF}; lanes 7 and 8, \textit{BP536ΔptlG}; lanes 9 and 10, \textit{BP536ΔptlI}. Positions of molecular size markers (in kilodaltons) are indicated at the left. The arrow indicates the protein band corresponding to PtlH. (C) Percentage of PtlH found in soluble fraction. Immunoblots from three independent experiments were analyzed by densitometry to determine the percentage of PtlH found in the soluble fraction. Results are reported as means ± standard deviations (error bars). An asterisk indicates significant \(P < 0.05\) differences between the percentages of PtlH found in the soluble fraction of the strain and that of either \textit{BP536} or \textit{BP536ΔptlA}. A plus sign indicates significant \(P < 0.05\) differences between the percentages of PtlH found in the soluble fraction of the strain and that of \textit{BP536ΔptlI}. Significant differences were determined by Tukey’s HSD test following ANOVA.
important clues as to when and how PT initially interacts with its transporter.

The finding that PT is required for proper localization of PtlH suggests that PtlH tightly engages with the transport apparatus only when PT is present. We found that neither the S1 subunit nor the B oligomer, composed of subunits S2, S3, S4, and S5, can substitute for the holotoxin. These results are consistent with and extend our previous finding, that secretion of PT from the bacterium requires assembly of the S1 subunit with subunits of the B oligomer (15). Importantly, these findings suggest that final assembly of the transporter does not occur in the absence of the holotoxin substrate.

Localization of PtlH in specific pti mutants provides further clues as to the events that may occur during Ptl transporter morphogenesis and the interaction of the transporter with PT. Mutants of B. pertussis lacking PtlD, PtlE, PtlF, and Ptg displayed the greatest abnormalities in PtlH localization, suggesting that these proteins may interact either directly with PtlH or indirectly, perhaps by stabilizing the protein(s) that interact with these proteins. These results are consistent with and extend our previous finding, that secretion of PT from the bacterium requires assembly of the S1 subunit with subunits of the B oligomer (15). Importantly, these findings suggest that final assembly of the transporter does not occur in the absence of the holotoxin substrate.

In this study, we also examined the requirement for ATP binding to PtlH and/or hydrolysis of ATP by PtlH for wild-type localization of PtlH. We believe that our result demonstrating a dependence of PtlH localization on ATP helps to shed light on the dynamics of the interaction of PtlH with the transporter-PT complex. In cells from which ATP has been deleted by the energy poisons CCCP or sodium arsenate, PtlH associates only loosely with the membrane, suggesting that normal interactions of PtlH with the membrane likely occur only if ATP is bound to the protein. These results are consistent with our finding that a mutant form of PtlH with alterations in its Walker A box also showed aberrant localization. Since the Walker A box of PtlH is essential for the ATPase activity of the protein and it is thought that this region is involved in nucleotide binding (38), this form of PtlH is predicted to be defective in ATP binding.

Savvides et al. (30) have demonstrated that HP0525, the Helicobacter pylori type IV transporter homolog of PtlH, can exist in three forms, an ATP-bound form, an ADP-bound form, and an apo form devoid of any nucleotides. These three forms differ in conformation with the largest differences observed between the apo form and the two nucleotide-bound forms of PtlH. The finding that PT but not PtlA is required for correct localization of PtlH to tightly engage with the transporter. Importantly, the finding that PT but not PtlA is required for correct localization of PtlH suggests that PT can interact with an incomplete form of the transporter that lacks PtlA.
forms. These workers suggest that, because of the similarity in conformation between the ADP- and ATP-bound forms, nucleotide binding is likely the critical step for biological activity and that ATP hydrolysis would serve only to accelerate nucleotide release, regenerating the apo form for another cycle of activity. Our results are consistent with a model in which binding of ATP to PtIH results in tighter association of PtIH with the membrane, likely through interactions with other protein members of the transport apparatus and/or PT. Hydrolysis of ATP to ADP and subsequent release of the nucleotide would convert PtIH back to the form that is only loosely associated with the membrane because it exhibits a lower affinity for the transport apparatus.

When taken together, our results on the effects of PT, Ptl proteins, and ATP on the localization of PtIH allow us to significantly extend our model of PT secretion (Fig. 9). In this model, toxin subunits are first individually secreted across the inner membrane into the periplasm. The periplasmic space contains the signal peptidase that would then cleave the signal sequences of each of the toxin subunits (26), followed by assembly of the toxin across the outer membrane.

and outer membranes of the bacterium, forming a translocation channel through which the substrate would pass (9). The substrates for other type IV transporters are cytoplasmic and are thought to interact with the transporter at the cytoplasmic face of the inner membrane through initial interactions with VirD4 (9), a protein that is absent in the Ptl system. Thus, current models of type IV transport do not easily accommodate a periplasmic substrate such as PT. The model shown in Fig. 9, which is based on the data presented in this study, depicts PT interacting with a partially assembled Ptl apparatus. Only after PT interacts with this subassembly would complete assembly of the apparatus occur. Such a model would explain how the periplasmic toxin is secreted by a transport apparatus that is believed to span the inner and outer membranes.

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