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Essential Role of the Consensus Nucleotide-Binding Site of PtlH in Secretion of Pertussis Toxin from *Bordetella pertussis*

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PtlH is a member of a specialized set of transport proteins that is essential for secretion of pertussis toxin (PT) from *Bordetella pertussis*. Previously, PtlH was shown to contain a consensus nucleotide-binding motif. Here, we demonstrate that introduction of plasmids containing mutant forms of *ptlH*, altered in the putative nucleotide-binding region, into a wild-type strain of *B. pertussis* resulted in inhibition of PT secretion. Thus, this region of PtlH appears to be essential for protein function. Moreover, the observed dominant negative phenotype suggests that PtlH either functions as a multimer or interacts with another component necessary for secretion of PT.

Pertussis toxin (PT) is one of the major virulence factors produced by *Bordetella pertussis*, the causative agent of the disease pertussis (whooping cough). After secretion from the bacterial cell, PT interacts with eukaryotic cells and catalyzes the ADP-ribosylation of a family of G proteins involved in signal transduction (8). Biological effects of PT include induction of lymphocytosis, increase in insulin secretion, and alteration of immune cell function (12, 20). PT is 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 (11, 13, 20). Each toxin subunit is synthesized with a signal sequence (11, 13, 20), suggesting that the individual subunits may utilize a Sec-like system to cross the inner membrane of this gram-negative bacterium. The mechanism by which the toxin traverses the outer membrane remained elusive until recently, when a specialized set of nine transport proteins, known as Ptl proteins, was shown to be essential for the secretion of PT from *B. pertussis* (5, 23). The *ptl* genes are located directly downstream from the *ptx* genes that encode the structural subunits of the toxin (Fig. 1) and are transcribed from the same promoter as the *ptx* genes (10). At this time, little is known about the Ptl proteins, but it is generally believed that they form a transport apparatus that facilitates the secretion of PT from the bacterial cells. The structure of the transport apparatus and the mechanistic details of the transport process remain to be elucidated.

One of the Ptl proteins, PtlH, which has been shown to be essential for PT secretion (23), is of particular interest since it contains a consensus nucleotide-binding motif (Walker A box) characteristic of certain nucleotide-binding proteins (22). The Walker A box region of nucleotide-binding proteins has been shown to interact directly with ATP or GTP and is therefore essential for the NTP-dependent functions of these proteins. In order to examine the role of the putative nucleotide-binding region of PtlH in secretion of PT, we have mutated *ptlH* in the region encoding the Walker A box motif and determined the effect of these mutations on PT secretion.

Cloning of *ptlH* and site-specific mutagenesis. The plasmids used in this study are listed in Table 1. To obtain a clone of *ptlH* that would be expressed in *B. pertussis*, we spliced *ptlH* to the 3′ end of a fragment of DNA containing the *ptx-ptl* promoter and the *ptxS1* region such that the ATG start codon of *ptlH* exactly replaced the ATG start codon of the *ptxS2* gene, which normally follows the *ptxS1* gene (Fig. 2). First, the promoter-*ptxS1* region was amplified by PCR in which the upstream primer contained a *Hin*dIII site followed by nucleotides 1 to 24 of the *ptx-ptl* region and the downstream primer consisted of the final 30 nucleotides of *ptxS1*, which included the *Xba*I site found at the end of this gene. This fragment was cloned into the *Hin*dIII-*Xba*I site of pALTER-1 vector (Promega, Madison, Wis.) to generate pSK08, which was transformed into *Escherichia coli* DH5α. The *ptlH* gene was then amplified by PCR in such a way that the intergenic region between *ptxS1* and *ptxS2* was added to the 5′ end of *ptlH* by utilizing an upstream primer that consisted of the *Xba*I site found at the end of *ptxS1* followed by the intergenic region between *ptxS1* and *ptxS2* and then by the first 24 bases of *ptlH* and a downstream primer that consisted of the final 21 nucleotides of *ptlH* followed by an *Sst*I site. This fragment was cloned into the *Xba*I-*Sst*I site of pSK08 to generate pSK09.

In order to determine whether the putative nucleotide-binding region of PtlH plays a role in protein function, we mutagenized *ptlH* at its Walker A box site. The putative nucleotide-binding region of PtlH is shown in Fig. 3. We chose to alter two amino acid residues of PtlH, G175 and K176, which are homologous to conserved residues in the Walker A box regions of nucleotide-binding proteins. These sites were chosen because alteration of analogous sites in the homologous proteins VirB11 of *Agrobacterium tumefaciens* (18) and XcpR of *Pseudomonas aeruginosa* (21) resulted in loss of function of these proteins. Site-directed mutagenesis of *ptlH* in pSK09 was carried out by using the Altered Sites II in vitro Mutagenesis

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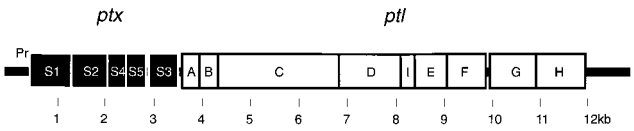

FIG. 1. Structure of the *ptx-ptl* region. Pr, *ptx-ptl* promoter.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference
B. pertussis 536	Nal^r Str^r derivative of Tohama I	19
pUFR047	Mobilizable, broad host range, Ap^r, Gm^r	4
pSK08	pALTER-1 vector containing promoter-*ptxS1* region	This study
pSK09	pALTER-1 vector containing promoter-*ptxS1* and *ptlH* regions	This study
pSK10	pUFR047 containing the promoter-*ptxS1* and *ptlH* regions	This study
pSK11	pUFR047 containing the promoter-*ptxS1* and mutated *ptxH* (K176A)	This study
pSK12	pUFR047 containing the promoter-*ptxS1* and mutated *ptxH* (G175S)	This study

system (Promega), according to the manufacturer's instructions. Oligonucleotides used for mutagenesis were GACCG GTTCGAGCAAGACCAC (Gly175 to Ser) and ACCGGTT CGGGCGCGACCACATTCATG (Lys176 to Ala), with the underlined portions of the oligonucleotides indicating changes from the wild-type sequence. Potential mutants were screened by single-stranded conformational polymorphism analysis (7). Mutations were confirmed by sequence analysis carried out according to the dideoxy method of Sanger et al. (17).

The *Hin*dIII-*Sst*I fragments containing the wild-type and mutated *ptlH* genes were individually cloned into the broad-host-range plasmid pUFR047, generating pSK10 (wild type), pSK11 (K176A mutant), and pSK12 (G175S mutant). These plasmids were transferred from *E. coli* into a wild-type strain of *B. pertussis* BP536 by conjugal transfer as previously described (10).

Effects of mutation of *ptlH* on secretion of PT from *B. pertussis*. Secretion of PT from wild-type *B. pertussis* strains harboring plasmids containing wild-type or mutant *ptlH* genes was first analyzed by visualizing the amount of S1 found in culture supernatants and in total cell cultures (cells plus supernatant) by using immunoblot analysis. As shown in Fig. 4, the S1 subunit could be detected in supernatants from BP536(pUFR047), which is a wild-type strain that normally produces and secretes PT and which harbors the broad-host-range plasmid pUFR047. Introduction of pSK10, which contains wild-type *ptlH*, into BP536 had no effect on the amount of S1 found in the culture supernatant. This plasmid contains not only the wild-type *ptlH* gene but also a copy of the *ptxS1* gene, which might be expected to result in overexpression of S1. While the supernatant did not contain increased levels of S1, the total culture (cells plus supernatant) did appear to contain an additional band which reacted with the S1 antibody and which migrated slightly slower than S1, indicating a higher molecular weight (Fig. 4). While the identity of this protein has not been unequivocally established, the possibility that this is a cellular form of S1 in which the signal sequence was not removed exists. Perhaps expression of S1 from both chromosomal and plasmid *ptxS1* genes results in high-level production of the protein that may overwhelm the normal processing capability of the cell.

BP536(pSK11) and BP536(pSK12), strains harboring plasmids encoding the mutant forms of *ptlH* in addition to expressing a wild-type copy of *ptlH* on the chromosome, secreted less S1 into the supernatant (Fig. 4) than either BP536(pUFR047) or BP536(pSK10), which express only the wild-type form of *ptlH*. While less S1 was observed in the culture supernatants from the strains containing plasmids encoding mutant forms of *ptlH*, the total culture (cells plus supernatant) of the mutant strains contained approximately the same amount of S1 as that of the strains expressing wild-type *ptlH*, suggesting that mutation of *ptlH* does not abolish production of PT but rather affects secretion of the toxin. Again, the higher-molecular-weight form of S1 was observed in the total cell culture (cells plus supernatant) of these mutants. In addition, a lower-molecular-weight form of S1 was observed in these fractions, and this form likely represents a breakdown product of S1 that is commonly observed (1) and may be found in the cultures of these strains, since S1 is not secreted and therefore may be readily available for degradation.

In order to confirm that the secretion of the S1 subunit observed by immunoblot analysis accurately represents secretion of the holotoxin molecule, we examined the ability of the culture supernatants from these strains to cluster Chinese hamster ovary cells, an effect that is due to PT intoxication of these cells (2, 6). As shown in Fig. 5, BP536(pUFR047) and BP536(pSK10) secreted comparable levels of biologically active PT. However, the strains harboring plasmids encoding the mutated forms of PtlH exhibited levels of biologically active PT in the culture supernatant that were 16 to 64 times lower than those seen with the wild-type strain.

Previously, we have shown that PtlH is essential for secretion of PT from *B. pertussis* (23). The results reported here provide additional information concerning PtlH function. First, our results demonstrate that alteration of amino acids in the putative nucleotide-binding domain of the protein results in an inactive form of the protein that can no longer support secretion of PT, findings consistent with the idea that this region plays a critical role in protein function, most likely by binding nucleotides. While we did not directly demonstrate the existence of the altered PtlH proteins in the strains containing the mutated *ptlH* genes, the observation of a dominant negative phenotype provides evidence that the altered PtlH proteins were produced in these strains in a form that is capable of interfering with the normal transport process. Presumably, it is the intact form of mutant PtlH that interferes with secretion; however, there exists the possibility that proteolytic fragments of the protein, if generated in sufficient quantities, could also interfere with secretion. Second, our finding that expression of

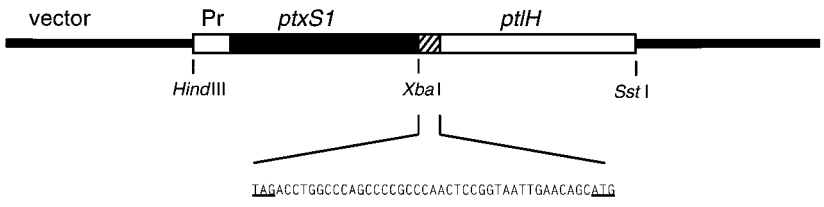

FIG. 2. Construction of plasmids capable of expressing *ptlH* in *B. pertussis*. The promoter-*ptxS1* region and *ptlH* were cloned into the broad-host-range vector pUFR047 as described in the text. The *ptlH* gene is spliced to *ptxS1* in such a way that *ptlH* would be driven by the *ptx-ptl* promoter (Pr). A detail of the region between *ptxS1* and *ptlH* shows the TAG stop codon of *ptxS1* (underlined), the nucleotides comprising the region between *ptxS1* and *ptxS2*, and the ATG start codon of *ptlH* (underlined).

Consensus:	G X X G X G K T
PtlH 170-177:	G Q T G S G K T
PtlH G175S:	G Q T G S **S** K T
PtlH K176A:	G Q T G S G **A** T

FIG. 3. Alteration of the putative nucleotide-binding region of PtlH. The consensus Walker A box region is shown along with the corresponding region from PtlH (amino acids 170 to 177). Shown in bold type are the changes in the protein that were made by site-specific mutagenesis in which amino acid 175 (glycine) was changed to serine and amino acid 176 (lysine) was changed to alanine.

the altered forms of PtlH interferes with the function of the wild-type protein expressed by the chromosomal copy of the gene suggests that the altered proteins can compete with the wild-type protein at essential sites of interaction. Whether this interaction is with other PtlH molecules to form oligomers or with other critical components of the secretory pathway (perhaps other Ptl proteins) remains to be elucidated.

PtlH is homologous to a number of proteins involved in secretion of macromolecules. The most closely related protein, VirB11, is involved in transport of nucleoprotein particles across the membranes of the plant pathogen *A. tumefaciens*. Mutations in the Walker A box of VirB11 result in loss of transport function (15, 18). While these mutations have not been found to exert transdominant effects, alterations in the Walker A box motifs of the homologous proteins PulE, EpsE, XcpR, and PilB exert dominant negative effects on the export of pullulanase from *Klebsiella oxytoca*, the secretion of cholera toxin from *Vibrio cholerae*, extracellular protein secretion in *P. aeruginosa*, and pilus assembly in *P. aeruginosa*, respectively (14, 16, 21). The biochemical roles of these proteins in transport processes have not been elucidated. VirB11 has been reported to exhibit ATPase activity and autophosphorylation activity (3), and EpsE has been shown to exhibit autophosphorylation activity (16).

In summary, PtlH is likely a nucleotide-binding protein that interacts with one or more critical components of the PT secretion apparatus. While additional experimentation is needed to confirm that PtlH is a nucleotide-binding protein, the presence of a classic nucleotide-binding motif and the finding that this region is critical for protein action suggest several potential functions for this protein in the transport process. Possibly, PtlH might provide energy for biogenesis of the transporter or for PT translocation by hydrolyzing ATP. Alternatively, PtlH might act as signaling protein, perhaps to signal the opening of a gate or channel, via kinase activity. Further studies of PtlH should elucidate the exact role that this protein plays in the secretion of PT from *B. pertussis.*

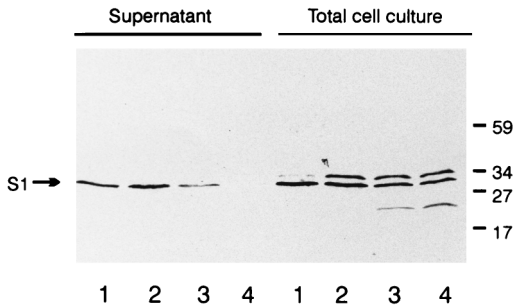

FIG. 4. Immunoblot analysis of culture supernatants and total cell cultures (cells plus supernatant) of *B. pertussis* Bp536 carrying broad-host-range plasmids either without or with wild-type or mutant forms of *ptlH*. Bp536 harboring plasmids containing wild-type and mutant forms of *ptlH* were grown to an A_{550} of 0.8 to 0.9 in Stainer Sholte medium containing 10 μg of gentamicin per ml. Supernatants (14 μl) or total cell culture (14 μl) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis as previously described (10). The S1 subunit was visualized with monoclonal antibody 3CX4 (9). Lane 1, BP536(pUFR047); lane 2, BP536(pSK10), plasmid containing wild-type *ptlH*; lane 3, BP536(pSK11), plasmid containing K176A *ptlH* mutant; lane 4, BP536(pSK12), plasmid containing G175S *ptlH* mutant.

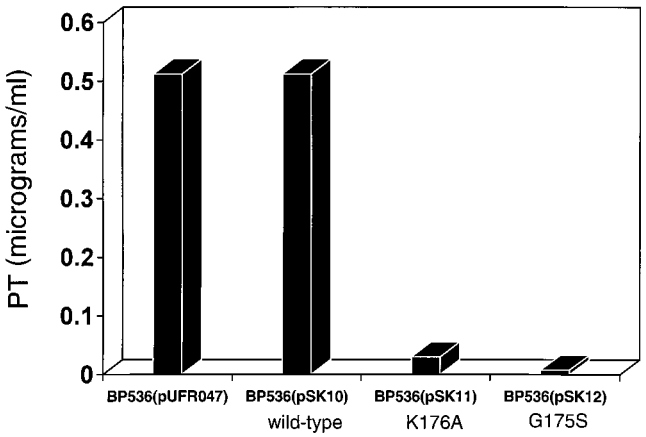

FIG. 5. Secretion of biologically active PT. Supernatants from strains grown as described in the legend to Fig. 4 were prepared. The amount of biologically active PT found in the culture supernatant of BP536 harboring plasmids encoding wild-type or mutant *ptlH* genes was determined by assessing the ability of serial dilutions of culture supernatants to induce the clustering of Chinese hamster ovary cells compared to a purified PT standard of known concentration as previously described (6).

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