The PtlE Protein of *Bordetella pertussis* Has Peptidoglycanase Activity Required for Ptl-Mediated Pertussis Toxin Secretion

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Pertussis toxin of *Bordetella pertussis* is secreted by a type IV secretion system comprised of the products of the nine *ptl* (pertussis toxin liberation) genes. These proteins are believed to form a complex spanning both the inner and outer membranes and passing through the peptidoglycan layer. Peptidoglycan acts as a barrier for transport through the periplasm of large folded molecules. Assembled pertussis toxin and the secretion component proteins PtlC through PtlH are too large to diffuse through intact peptidoglycan. Therefore, we hypothesized that the Ptl system contains a peptidoglycanase activity. The PtlE protein was found to exhibit a sequence match to the active site of glycohydrolase enzymes. An N-terminally polyhistidine-tagged PtlE fusion protein, constructed and expressed in *Escherichia coli* and in *B. pertussis*, exhibited peptidoglycanase activity on activity gels. A fusion protein with alanine substitutions at the putative active site residues (aspartic acid at position 53 and glutamic acid at position 62) lacked peptidoglycanase activity. *B. pertussis* strains with the amino acid substitutions were deficient for pertussis toxin secretion. Based on these results, we concluded that PtlE is a peptidoglycanase responsible for the local removal or rearrangement of the peptidoglycan layer during Ptl secretion complex assembly.

Pertussis toxin is the most complex toxin yet discovered. It is an AB₅ toxin comprised of the products of five genes, S1 through S5 (17, 22). The A subunit of the toxin is the S1 polypeptide, while the pentameric B subunit is comprised of S2, S3, S4, and S5 assembled in a ratio of 1:1:2:1 (26). Secretion of pertussis toxin past the outer membrane of *Bordetella pertussis* requires the nine *ptl* (pertussis toxin liberation) genes (8). The *ptl* genes are located immediately downstream of the pertussis toxin genes, transcribed from the same promoter (7, 31). Based on homology to other type IV systems, such as the *Agrobacterium tumefaciens* *virB* operon, and to the P-plasmid *tra* conjugation genes (32), it is predicted that the Ptl proteins form a large complex spanning both the inner and outer membranes. While they do not share homology, the type IV complexes are comparable to the flagellar basal body or the type III secretion systems, which also span the cytoplasm and cross the peptidoglycan barrier. During assembly of the flagellum, flagellar subunits are secreted from the bacterium through the basal body (1). Likewise, proteins involved in pathogenesis are secreted into the extracellular milieu through the flagellar export apparatus of *Salmonella enterica* serovar Typhimurium (33).

Pertussis toxin is assembled in the periplasmic space, and the Ptl system mediates secretion from the periplasm past the outer membrane. However, the Ptl secretion system appears to span the inner membrane as well as the outer membrane since Ptl proteins have been found localized to both the inner and outer membranes (13). It is thought that the energy to power secretion comes from the cytoplasm. The PtlC and PtlH pro-

teins contain ATP binding domains that are required for secretion (6, 14) and are predicted to be localized to the cytoplasm, where they could transduce energy to the membrane-bound components of the secretion system. The secretion complex must span the peptidoglycan layer. Since peptidoglycan forms a rigid barrier around the cell (9), a means of removing or rearranging this portion of the peptidoglycan must be available. Recently, peptidoglycanase proteins involved in local rearrangements of peptidoglycan have been found in other systems involving passage through the membrane. An accessory protein of the flagellar basal body, FlgJ, of *S. enterica* serovar Typhimurium has been found to have peptidoglycanase activity, which is required for motility (21). Peptidoglycan hydrolase homology has been identified in the VirB1 protein of the *A. tumefaciens* *virB* type IV secretion system (20) that has been shown to enhance tumorigenesis (16). Similarly, several bacteriophages have been found to have lytic transglycosylase activities thought to be involved in the invasion step. These enzymes include GP16 of T7 (19) and the P7 structural protein of PRD1 (25). The activity involved in phage entry is different from the activity involved in lysis of an infected bacterium in that it affects the peptidoglycan structure only locally and does not promote cell lysis.

This study was carried out to identify a peptidoglycanase in the Ptl system. PtlE was chosen as the candidate peptidoglycanase because it was found to possess sequence similarity to the active site of glycohydrolase enzymes at its N terminus using the program BLOCKS (11). We showed that a polyhistidine-tagged PtlE has peptidoglycanase activity. Mutants with amino acid substitutions at the putative glycohydrolase active site lacked activity and exhibited significantly reduced secretion of pertussis toxin. On the basis of these results, we suggest that PtlE is a peptidoglycanase specific to the pertussis toxin secretion apparatus.

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant features	Source or reference
<i>E. coli</i> strains		
BL21(DE3)(pLysS)	T7 polymerase	Invitrogen
DH5 α	High-efficiency transformation, Nal ^r	Gibco BRL
UT5600	OmpT ⁻ <i>mlt-1</i>	New England Biolabs
<i>B. pertussis</i> strains		
BP338	Tohama I derivative, Nal ^r	30
BPRA	Deletion of <i>ptx/ptl</i> promoter and genes for S1 through S5, Nal ^r Strep ^r	2
Plasmids		
Commercial vectors		
pBluescript KS+	Cloning vector, Amp ^r	Stratagene
pCR2.1	TA cloning vector, Amp ^r Kan ^r	Invitrogen
pRSETB	Expression vector, T7 promoter, polyhistidine metal-binding domain, Amp ^r	Invitrogen
pET-21b	Expression vector, T7 <i>lac</i> promoter, Amp ^r	Novagen
pMAL-p2x	Maltose-binding protein fusion vector	New England Biolabs
His tagging of PtlE		
pKC34	<i>ptx/ptl</i> operon in pSP72, Amp ^r	8
pAR186	<i>ptlE</i> in pRSETB	This study
pAR253-1	<i>ptlE</i> D53A in pRSETB	This study
pAR278-1	<i>ptlE</i> E62A in pRSETB	This study
pAR278-2	<i>ptlE</i> D53A E62A in pRSETB	This study
pAR312-1	<i>ptlE</i> (1–84) ^a in pET-21b	This study
pAR312-2	<i>ptlE</i> D53A (1–84) in pET-21b	This study
pAR312-3	<i>ptlE</i> E62A (1–84) in pET-21b	This study
pAR312-4	<i>ptlE</i> D53A E62A (1–84) in pET-21b	This study
Expression in <i>B. pertussis</i>		
pBBR1-MCS2	Broad-host-range cloning vector, Kan ^r	15
pCW211-5	Plasmid containing <i>B. pertussis</i> <i>cpn10</i> promoter	29
pAR228-1	pBBR1-MCS2 with the <i>B. pertussis</i> <i>cpn10</i> promoter inserted between the <i>SacI</i> and <i>SacII</i> sites	This study
pAR249-1	His-tagged <i>ptlE</i> in pAR228-1	This study
Cloning of <i>ptlE</i> mutants		
pAR146	<i>NotI</i> -to- <i>HindIII</i> fragment from pKC34 containing <i>ptlE</i> through <i>ptlH</i> genes in pBluescript	This study
pKC109	<i>oriT</i> Gent ^r , <i>cycA</i> fragment	8
pAR287-2	D53A mutation in pKC34	This study
pAR264-3	E62A mutation in pKC34	This study
pAR264-4	D53A and E62A mutations in pKC34	This study

^a Amino acid positions are shown in the parentheses.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used are shown in Table 1. *Escherichia coli* strains were grown on L agar. *B. pertussis* strains were grown on Bordet-Gengou (BG) agar or in Stainer-Scholte (SS) broth overlaid onto BG agar as previously described (8, 30).

Peptidoglycanase activity gels (zymograms). Peptidoglycanase activity was detected by using the methods of Potvin et al. (23) and Buist et al. (5). Bacterial cells were separated in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels containing 0.2% (wt/vol) autoclaved, lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells (Sigma). Proteins were allowed to renature in the gels by shaking the gels in 25 mM Tris-HCl (pH 7.5)–1% (vol/vol) Triton X-100 for 48 h, with several changes of buffer to remove the SDS. Peptidoglycanase activity resulted in the formation of clear bands. Gels were stained for 5 min with 1% methylene blue in 0.1% (wt/vol) KOH to increase contrast and were destained in three to four changes of deionized water. As a further refinement of the procedure, the gel shown in Fig. 8 was renatured in 25 mM Tris-HCl (pH 7.5)–1% (vol/vol) Triton X-100 for 24 h and then in 25 mM Tris-HCl (pH 5.0)–1% (vol/vol) Triton X-100 for an additional 24 h. The optimum pHs for lysozyme and the two major *E. coli* transglycosylases are in the vicinity of pH 5, so it was thought that decreasing the pH in the final step would increase the activity of the protein.

Western blotting. Western blotting was performed as previously described (8). Samples were mixed at a 1:1 ratio with sample buffer. Proteins were separated on 12% acrylamide SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were probed with anti-polyhistidine antibody (Sigma) or anti-PtlF antibody.

Generation of PtlF antibody. Hydropathy and secondary-structure data predict that the region of PtlF from amino acid residues 73 to 205 has the highest antigenicity index (12). This region was amplified by PCR by using primers 5-F80 and 3-F80. These primers introduced an *XbaI* restriction site into the 5' end and an *HindIII* restriction site into the 3' end. The PCR product was cloned into TA cloning vector pCR2.1 by using a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) according to the manufacturer's directions. The PCR product was ligated into plasmid pMAL-p2x by using the *XbaI* and *HindIII* sites. A maltose-binding protein fusion to this region of PtlF was generated and purified by using the pMAL protein fusion and purification system (New England BioLabs, Inc.). The fusion protein was expressed in *E. coli* UT5600 to increase expression levels and decrease proteolytic degradation. Rat polyclonal antibodies to the fusion protein were generated at Harlan Bioproducts for Science (Indianapolis, Ind.). Rats were injected with 200 μ g of fusion protein with complete Freund's adjuvant at zero time, and this was followed by two boosts consisting of 100 μ g with incomplete Freund's adjuvant on days 28 and 56. The final bleed was done on day 70.

Separation of membrane and soluble proteins. *B. pertussis* BP338(pAR249-1) was grown in SS broth overlaid onto BG plates as described above. Ten milliliters of a 24-h culture was pelleted by centrifugation, washed two times in 10 ml of 4°C Tris-NaCl (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA), and resuspended in 10 ml of Tris-NaCl with 20 μ l of Sigma protease inhibitor cocktail (product no. P 8465). Cells were broken by sonication for 8 min in a Branson 2510 sonicating water bath in 2.5-ml aliquots. Sonicated cultures were centrifuged at 9,000 $\times g$ for 10 min to remove unbroken cells. Samples were then centrifuged at 100,000 $\times g$ for 1 h to separate the membrane and soluble fractions. Membranes were resuspended in 1 ml of Tris-NaCl by sonication for 1

TABLE 2. Primers used

Primer	Sequence ^a
5-F80	GGCTCTAGAGACGGCTGGCAATTCAGCC
3-F80	CAGAAGCTTACCCGGTCTGAACGTGAGCC
5-E180	GGCTCTAGAATGGGCCATCCTGGCCATC
3-E280	CAGAAGCTTCATGGCTGTCCAGCCTCCG
5-HSP	GAGCTCGCGAAGACCCGCC
3-HSP	CCGCGGATGAGGAACCTCTG
5-AR156	CTTGACGCCTGCCAGACGC
3-AR156	GTCTGGGCAGGCGTCAAGGGG
5-AR264	GCCGCGGCGCGCGGTGGAC
3-AR264	GCGGCCGCGGCATGCCCG
3-AR301	GAAGCTTACCATGCACGGCGTTCCGAC
5-D80	GGCTCTAGAGCGGGCTGCGGCG

^a The underlined nucleotides are the nucleotides altered to produce the amino acid substitutions.

min. Soluble fractions were precipitated with 20% (wt/vol) trichloroacetic acid and resuspended in 1 ml of Tris-NaCl.

Construction of fusion proteins for overexpression in *E. coli*. The *PtIE* gene was cloned out of pKC34 by using primers 5-E180 and 3-E180, which added the *Xba*I restriction enzyme site to the 5' end and the *Hind*III restriction enzyme site to the 3' end. The PCR product was TA cloned into pCR2.1 as described above and then ligated into expression vector pRSETB at the *Nhe*I and *Hind*III sites, resulting in an N-terminal polyhistidine fusion, plasmid pAR186 (Table 1).

Overexpression of fusion proteins in *E. coli*. The polyhistidine-tagged *PtIE* fusion proteins were expressed in *E. coli* BL21[DE3](pLysS). Cultures were grown in L broth containing ampicillin and chloramphenicol at appropriate concentrations. Single, freshly transformed colonies were added to 10 ml of L broth and incubated overnight without shaking at 37°C. The overnight culture (0.2 ml) was used to inoculate 10 ml of fresh L broth, which was incubated with shaking at 37°C until the mid-log phase of growth. Expression of the fusion protein was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cultures were incubated for an additional 2 h and harvested in 1-ml aliquots by centrifugation. Cell pellets were resuspended to an optical density at 600 nm (OD₆₀₀) of 4 for analysis.

Construction of fusion proteins for overexpression in *B. pertussis*. A vector for the expression of fusion proteins in *B. pertussis* was constructed in the broad-host-range cloning vector pBBR1-MCS2 (15). Primers 5-HSP and 3-HSP were used to amplify the *B. pertussis* *cpt10* promoter (10) from pCW211-5 (29) and to add the *Sac*I restriction enzyme site to the 5' end and the *Sac*II restriction enzyme site to the 3' end. The promoter was ligated into pBBR1-MCS2 at the *Sac*I and *Sac*II sites to create pAR228-1. This placed the promoter immediately upstream of the multiple cloning region. The His-tagged fusion protein gene was ligated into the *B. pertussis* expression vector pAR228-1 at the *Xba*I and *Hind*III sites to generate plasmid pAR249-1. This plasmid was electroporated into *B. pertussis* BP338 by using the procedure developed by Weingart et al. (29). Colonies were selected with kanamycin, and individual transformants were grown out on BG agar plates at 37°C for 48 h.

Construction of amino acid substitutions in *PtIE*. Point mutations D53A and E62A and a double mutation (D53A E62A) were constructed by PCR by using the primers listed in Table 2. The D53A mutation was made by using primers 5-AR156 and 3-AR156. The E62A mutation was made by using primers 5-AR264 and 3-AR264. The nucleotides altered to produce the amino acid substitutions are indicated in Table 2. The double mutation was constructed by using primers 5-AR264 and 3-AR264 and a plasmid containing the PCR fragment with the D53A mutation as the template. Outside primers 5-D80 and 3-F80 were used to clone the mutations into the *ptc/ptl* operon. The PCR products were TA cloned into pCR2.1. The cloned point mutations were ligated into pAR146 by using *Not*I and *Aat*II. The *Not*I-to-*Hind*III region from the resulting plasmid was then ligated back into the remaining portion of pKC34 to regenerate the complete operon containing the desired nucleotide substitution(s), as shown in Fig. 1. The *Hind*III fragment of pKC109 containing the gentamicin resistance gene, the origin of transfer, and an internal fragment of the adenylate cyclase gene was ligated into these plasmids to make suicide vectors (for D53A, pAR287-2; for E62A, pAR264-3; and for D53A E62A, pAR264-4) for triparental mating, as shown in Table 1. The suicide plasmids were transformed into *E. coli* HB101.

Vectors for expression of His-tagged *ptIE* in *E. coli* (pAR317-2, pAR317-3, and pAR317-4) containing amino acid substitutions D53A and E62A and the double

FIG. 1. Features of the *PtIE* protein. The putative active site residues and membrane-spanning region are indicated.

mutation were generated in the manner described above for plasmid pAR186 by using plasmids pAR287-2, pAR264-3, and pAR264-4 as the templates for PCR. C-terminal truncations of the tagged wild-type and mutant *ptIE* genes were subcloned from the full-length genes by using the T7 promoter primer and primer 3-AR301. The PCR products were TA cloned and then ligated into expression vector pET-21b at the *Nde*I and *Hind*III sites to produce plasmids pAR253-1, pAR278-1, pAR278-2, and pAR312-4. These plasmids expressed the N-terminal portion of *PtIE* and the *PtIE* point mutations up to amino acid 84.

Introduction of mutations into the *B. pertussis* chromosome. Each plasmid containing a mutant *ptIE* or the wild-type gene was integrated into the chromosome of *B. pertussis* via a region of adenylate cyclase homology as described previously (8). Triparental matings were performed as described by Barry et al. (3). The mating mixtures contained 250 μl of *E. coli* HB101 containing one of the suicide plasmids and 250 μl of an *E. coli* strain containing helper conjugative plasmid pUW956 (30) suspended in L broth added to 2 ml of *B. pertussis* in SS broth. The mating mixtures were plated on SS medium plates containing 40 mM MgCl₂ and incubated at 37°C. After 6 h, the mixtures were streaked on BG agar plates with nalidixic acid and gentamicin to select for single recombination events where the plasmid had integrated into the *B. pertussis* chromosome, and they were further screened for recombination into the adenylate cyclase locus by selecting nonhemolytic colonies.

Toxin secretion assay. Single colonies from independent triparental matings were streaked onto BG agar and incubated for 48 h at 37°C. A suspension of each culture in SS broth with an OD₆₀₀ of 0.1 was made, and 7 ml of the suspension was overlaid onto a BG agar plate with gentamicin and nalidixic acid and incubated for 24 h at 37°C. Cells were harvested in 1-ml aliquots by centrifugation. Supernatant samples were filter sterilized to remove the remaining bacteria. Cell pellets were washed in 1 ml of phosphate-buffered saline (PBS) (pH 7.4). For Western blot analysis, pellets were resuspended in PBS to an OD₆₀₀ of 8. For determination of periplasmic toxin, bacterial pellets were resuspended in 100 μl of a 20-mg/ml lysozyme solution and incubated for 30 min at 37°C. The reaction was stopped by addition of 900 μl of PBS-0.05% (vol/vol) Tween 20. Samples were centrifuged to pellet cell debris, and each supernatant (periplasmic fraction) was filter sterilized.

CHO cell assay. Chinese hamster ovary (CHO) cell assays were performed as described previously (8). Briefly, serial twofold dilutions of samples were made in Ham's F-12 medium containing 1% fetal bovine serum. Samples were added to CHO cell monolayers in 96-well plates and incubated for 48 h at 37°C in a tissue culture incubator. Pertussis toxin causes CHO cells to lose focal adhesion and produces a clusters-of-grapes morphology. Cell morphology in wells containing test samples was compared to cell morphology in wells containing twofold dilutions of purified pertussis toxin (List Biologicals, Campbell, Calif.) and cell morphology in control wells with no toxin. Each sample was assayed in duplicate with at least three independent repeats. The limit of detection for purified pertussis toxin was determined on each plate, and the last positive well of the test samples was assigned that value. The standard error of the mean was graphed, and the Student *t* test was used to determine statistical significance.

RESULTS

***PtIE* has sequence homology to glycohydrolases.** The *ptIE* gene is one of the nine *ptl* genes located downstream of the pertussis toxin genes and required for the export of folded pertussis toxin past the outer membrane of the bacterium. The *PtIE* protein is predicted to be 276 amino acids long with a molecular mass of 30 kDa (Fig. 1). It contains a single membrane-spanning domain (amino acids 84 to 127), which we have predicted to be oriented with the N terminus on the periplasmic side of the inner membrane using TMPred (<http://www.ch.embnet.org>). A preliminary search performed with the program BLOCKS identified a region of homology to glycosyl

FIG. 2. Analysis of polyhistidine-tagged PtIE expressed in *E. coli* BL21. Lane 1, uninduced BL21(pAR186); lane 2, BL21(pAR186) induced with IPTG. (A) Western blot analysis with anti-polyhistidine antibody; (B) zymogram analysis showing peptidoglycanase activity.

hydrolase family 9 (11). This region of homology spans amino acids 51 through 76 of PtIE and is predicted to be localized to the periplasmic space. The most conserved region contains two acidic amino acids, located nine residues apart (Fig. 1, residues D53 and E62). These residues, an aspartic acid and a glutamic acid residue, have been shown to be catalytically important in another member of this family (28).

Peptidoglycanase activity of PtIE. Peptidoglycanase activity was monitored by using activity gels. Bacterial proteins were separated on SDS-PAGE gels containing *M. lysodeikticus* cells as a source of peptidoglycan. Following electrophoresis, protein was allowed to renature, and peptidoglycanase activity was detected as uncolored bands following staining with methylene blue. In initial studies, no differences were detected between wild-type *B. pertussis* and BPRA, the pertussis toxin and PtI deletion mutant. The failure to detect this activity in *B. pertussis* was not unexpected and was likely due to the need to tightly regulate this potentially dangerous activity.

Construction and expression of fusion proteins. To improve sensitivity, PtIE was overexpressed as a polyhistidine-tagged fusion. PtIE has two potential initiation codons. Since it is not known which of these codons is the actual initiation site of PtIE, the first possible start site was chosen for the fusion protein to ensure that the entire protein would be included in the resulting fusion protein. The PtIE gene was cloned out of pKC34 by PCR using primers 5-E180 and 3-E180 (Table 2), which added the *Xba*I restriction enzyme site to the 5' end and the *Hind*III restriction enzyme site to the 3' end, and ligated into expression vector pRSETB at the *Nhe*I and *Hind*III sites, resulting in an N-terminal polyhistidine fusion.

The polyhistidine-tagged PtIE protein was overexpressed in *E. coli*. This fusion protein migrated at 30 kDa, as determined by Western blotting using antibody to polyhistidine (Fig. 2A). Peptidoglycanase activity was monitored by using a peptidoglycanase activity gel. Peptidoglycanase activity comigrated with the fusion protein (Fig. 2B, lane 2). No peptidoglycanase activity was detected at that molecular weight in the uninduced control (Fig. 2B, lane 1).

Expression of the PtIE fusion protein in *B. pertussis*. For expression in *B. pertussis*, vector pAR228-1 was constructed by

FIG. 3. Analysis of polyhistidine-tagged PtIE expressed in *B. pertussis* BP338. (A) Western blot analysis with anti-polyhistidine antibody. Lane 1, cells; lane 2, soluble fraction; lane 3, membrane fraction. (B) Zymogram analysis. Lane 1, soluble fraction; lane 2, membrane fraction.

inserting the *B. pertussis* *cpn10* promoter into the broad-host-range cloning vector pBBR1-MCS2, which is capable of replication in *B. pertussis*. The region encoding the fusion protein was cut out of the pRSETB vector by using *Xba*I and *Hind*III and ligated into the *B. pertussis* expression vector to create pAR249-1.

The His-tagged PtIE fusion protein was expressed by wild-type *B. pertussis* strain BP338, as determined by Western blotting (Fig. 3A). The major cross-reacting band detected by antibodies to polyhistidine was observed to migrate at 25 kDa, slightly faster than the same fusion protein expressed in *E. coli*. Since the fusion protein is N terminally tagged, this suggests that the C terminus of the PtIE protein may be processed in *B. pertussis*.

The native PtIE protein has been shown to localize to the membrane fraction in *B. pertussis* (13). In order to determine where the fusion protein was localized, *B. pertussis* BP338 (pAR249-1) expressing His-tagged PtIE was separated into soluble and membrane fractions by sonication and high-speed centrifugation. Samples of the fractions were analyzed by using Western blotting and a peptidoglycanase activity gel. The fusion protein was found to localize to the membrane fraction by Western blotting (Fig. 3A). The PtIE fusion protein in the membrane fraction was also found to retain peptidoglycanase activity (Fig. 3B).

Construction of *ptlE* point mutants within the *ptx/ptl* operon. The *ptl* genes have overlapping reading frames. The stop codon of the *ptlI* gene overlaps codons 50 and 51 of the *ptlE* gene, and the stop codon of the *ptlE* gene overlaps the start codon of the *ptlF* gene (Fig. 4). Attempting to knock out the *ptlE* gene for

FIG. 4. Cloning of amino acid substitutions into the *ptx/ptl* operon. The arrows represent primers used to produce PCR products. The asterisks represent bases that were altered.

FIG. 5. Western blot of PtIF expression for the wild type and PtIE mutants. The *ptx/ptl* operon containing wild-type PtIE or point mutants was introduced into the chromosome of pertussis toxin deletion strain BPRA at the adenylate cyclase toxin locus. Expression of PtIF was assessed to monitor polarity. Lane 1, BPRA; lane 2, BPRA with wild-type (WT) PtIE (from pKC34); lane 3, BPRA with PtIE D53A (from pAR287-2); lane 4, BPRA with PtIE E62A (from pAR264-3); lane 5, BPRA PtIE D53A E62A (from pAR264-4).

analysis by complementation is therefore impractical, and another strategy had to be used to test the effect of the point mutations on toxin secretion.

Individual amino acid substitutions D53A and E62A were made at the putative active site residues by PCR. A double mutant (D53A E62A) was also constructed. The amino acid substitutions were inserted into the *ptlE* gene within the *ptx/ptl* operon by PCR, as diagramed in Fig. 4. Each plasmid containing a mutant *ptlE* or the wild-type gene was integrated into the chromosome of *B. pertussis* via a region of adenylate cyclase homology, as described previously (8). For haploid analysis, plasmids were integrated into the chromosome of BPRA, a *ptx/ptl* mutant strain containing a deletion of the *ptx/ptl* promoter and the S1, S2, S4, and S5 genes (2). For merodiploid analysis, wild-type strain BP338 was used.

To ensure that the amino acid substitutions had not resulted in polar mutations, immunoblot analysis was performed on the haploid mutant strains by using an antibody to PtIF, the product of the gene immediately downstream of the *ptlE* gene. All three mutant plasmids and the control wild-type plasmid restored expression of PtIF in BPRA, and similar levels of expression were observed for all strains (Fig. 5).

***B. pertussis* *ptlE* mutants are defective for toxin secretion.** CHO cells were used to determine the concentrations of active toxin in samples of culture supernatant (secreted toxin) and cell lysates (periplasmic toxin). Integration of the wild-type *ptx/ptl* operon into BPRA restored toxin expression and secretion, as previously demonstrated (8). Secretion was reduced by more than 70% in all three mutants compared to the wild-type control strain (Fig. 6), and the differences were statistically significant. The defect in secretion did not result in increased levels of periplasmic toxin, a result that has been observed for several other *ptl* mutants (8).

Mutations that disrupt proper folding or localization of a protein that is part of a complex can result in dominant negative effects. A previous mutant of PtIE was found to have a dominant negative phenotype. The mutants from this study

FIG. 6. Toxin expression and secretion in haploid strains (*ptx/ptl* mutant background). PTWT, BPRA with wild-type PtIE (from pKC34); D53A, BPRA with mutant PtIE from pAR287-2; E62A, BPRA with mutant PtIE from pAR264-3; D53A+E62A, BPRA with mutant PtIE from pAR264-4. Cell-associated (periplasmic) and secreted toxins were quantified by using the CHO cell activity assay. Secretion of toxin was significantly reduced in strains expressing mutant *ptlE* genes, including D53A ($P < 0.008$), E62A, and the double mutant ($P < 0.003$) ($n = 4$).

were therefore tested for dominant negative effects by integrating the mutant PtI operon into the adenylate cyclase toxin gene of wild-type strain BP338. The amino acid substitutions did not affect toxin expression or secretion in the wild-type background (Fig. 7), suggesting that these mutants do not have a dominant negative phenotype.

Amino acid substitutions at the glycohydrolase active site residues reduce peptidoglycanase activity. To demonstrate that the loss of secretion was due to the loss of glycohydrolase activity in PtIE, His-tagged *ptlE* genes containing the D53A, E62A, and D53A plus E62A amino acid substitutions were expressed in *E. coli* by using truncated *ptlE* genes expressing only the N-terminal domain of the protein (through residue 84) to improve the solubility of the protein and allow higher levels of overexpression. The recovery of mutant or wild-type protein was similar in all cases, as determined by Western blotting (Fig. 8A). The proteins were tested for peptidoglycanase activity by using the activity gel assay (Fig. 8B) and gels

FIG. 7. Toxin expression and secretion in merodiploid strains (wild-type background). PTWT, BP338 with a second copy of wild-type PtIE (from pKC34); D53A, BP338 with a second copy of mutant PtIE from pAR287-2; E62A, BP338 with a second copy of mutant PtIE from pAR264-3; D53A+E62A, BP338 with a second copy of mutant PtIE from pAR264-4. Cell-associated (periplasmic) and secreted toxins were quantified by using the CHO cell activity assay. Periplasmic and secreted toxin levels were not significantly different for any of the strains tested.

FIG. 8. Activity of polyhistidine-tagged mutant PtlE proteins expressed in *E. coli* BL21[DE3]. Lane 1, vector control (pET-21b); lane 2, His-tagged PtlE wild type (pAR312-1); lane 3, His-tagged PtlE D53A (pAR312-2); lane 4, His-tagged PtlE E62A (pAR312-3); lane 5, His-tagged PtlE double mutant (pAR312-4). (A) Western blot analysis with anti-polyhistidine antibody; (B) zymogram analysis.

loaded with the same amount of protein as the Western blot. Peptidoglycanase activity was detected with the wild-type protein but not with the mutant proteins (Fig. 8B).

DISCUSSION

The peptidoglycan of gram-negative bacteria is thought to restrict passage of folded proteins larger than 25 kDa through the periplasm (24). Local cleavage of peptidoglycan has been shown to be essential for the formation of large transmembrane complexes in a number of diverse systems of gram-negative bacteria, including flagellar assembly (21), conjugation (4), and phage entry into the cytoplasm (19, 25).

Soon after the sequence of the Ptl operon was completed (31), we identified PtlE as a possible candidate for performing this function in pertussis toxin secretion when it was found to match a family of glycohydrolases using the program BLOCKS (11). While the overall match was very poor, there was conservation of acidic residues known to be essential for catalytic activity. Interestingly, as more sequence data has become available, the PtlE glycohydrolase match is no longer reported using this program. In addition, a Blast search using amino acids 1 through 84 of PtlE does not yield any significant matches.

The peptidoglycanases, which include lysozymes and transglycosylases, have been grouped into several families based on low levels of homology in the region of the active site groove. However, the level of similarity across families is too low to establish a consensus sequence (27). In spite of the low sequence homology, the peptidoglycanases that have been crystallized have had remarkably similar structures in the region of the catalytic fold (27). All of the families of peptidoglycanases have a catalytic glutamic acid residue in the active site, and some glycanases also have a catalytic aspartic acid residue. These residues are essential for the glycanase activity (18).

Each of the Ptl proteins has a strong match to proteins in the VirB operon of *A. tumefaciens*. The VirB operon promotes the

transfer of DNA from the bacteria into a plant cell utilizing a large protein complex that appears to be similar to the Ptl secretion complex (32). The *ptlE* gene and its homolog, *virB8*, have 50% amino acid similarity in the C-terminal region but have no significant similarity in N-terminal region (amino acids 1 to 102 of PtlE and amino acids 1 to 53 of VirB8). Homology to glycohydrolases has been identified in the VirB system (20); however, the match is in the *virB1* gene. There is no homolog of VirB1 in the *ptl* operon. These results suggest that in spite of the high level of overall homology between these proteins, the N-terminal domains of PtlE and VirB8 likely perform different functions.

We have demonstrated that PtlE, a protein essential for secretion of pertussis toxin (8), also possesses peptidoglycanase activity, and this activity is essential for pertussis toxin secretion. Local removal of peptidoglycan is needed for formation of the transmembrane secretion complex. We suggest that this mechanism is universal and that all type III and type IV secretion systems require a peptidoglycanase activity for assembly.

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