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Pertussis Toxin Export Genes Are Regulated by the *ptx* Promoter and May Be Required for Efficient Translation of *ptx* mRNA in *Bordetella pertussis*

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The gene products from an 8-kb region adjacent to the 3′ end of the *ptx* operon are required by *Bordetella pertussis* for the export of pertussis holotoxin. At least one of these gene products (PtlC) is specifically required for the export of assembled holotoxin from the periplasmic space. *ptlC* mutants exhibit a 20-fold reduction in the amount of holotoxin present in the culture supernatant but have no effect upon the assembly or steady-state level of holotoxin present in the periplasmic space. Impaired export of holotoxin from the *ptlC* strain blocks expression of toxin at a posttranscriptional level, and wild-type levels of *ptx* mRNA are detected in the mutant strain. The transcription of *ptl* is subject to modulation by $MgSO_4$ in the same manner as *ptx* is; however, in *B. pertussis* strains containing an *E. coli tac* promoter in place of the native *ptx* promoter, wild-type levels of *ptx* mRNA are present and holotoxin is synthesized and exported even in the presence of $MgSO_4$. Promoter mapping of the region extending from the *ptxS3* coding region to the *ptlC* coding region failed to detect the *ptl* transcription initiation site. Additional RNase protection experiments with *ptx* promoter deletion and substitution strains indicate that the *ptl* operon is transcribed from the *ptx* promoter as part of a >11-kb mRNA.

The hexameric pertussis toxin (PT) protein is one of the most structurally complex bacterial toxins known. The enzymatically active A monomer (or S1 subunit) is associated with the B oligomer, which contains the S2, S3, S4, and S5 subunits complexed in a 1:1:2:1 molar ratio (25). The genes for these subunits have been cloned and sequenced (8, 13). Each of the subunits is thought to be translated from the polycistronic *ptx* mRNA as a precursor protein containing a signal sequence. The individual subunit precursors appear to be separately processed and translocated across the cytoplasmic membrane into the periplasmic space where they are assembled into mature holotoxin. The assembled holotoxin is then somehow translocated from the periplasm across the outer membrane and exported. A transposon insertion in *ptxS3* results in the accumulation of the other subunits in the periplasmic space of the mutant strain (9, 12). The inability to detect any individual subunits in the culture medium of the *ptxS3* insertion mutant indicates that the unassembled subunits are not transported across the outer membrane. Inefficient assembly or export of the B oligomer occurs in the absence of an intact S1 subunit; several different mutations in the S1 subunit (including carboxy-terminal deletions which prevent association with the B oligomer) give rise to strains that export low levels of the B oligomer into the culture medium (1, 14).

The expression of *ptx* and other virulence factors is subject to regulation by the *bvgA* and *bvgS* gene products of *Bordetella pertussis* (18, 23). These proteins act similarly to other two-component signal transducing pathways (10) to activate the transcription of *ptx*, *fhaB*, *cyaA*, and other genes encoding virulence factors in *B. pertussis*. The inability of the *bvg* gene products to activate expression of *ptx* in *Escherichia coli* has led to the speculation that expression of *ptx* may require factors in addition to *bvg* (11); however, the processing events and factors required for the expression, assembly, and export of pertussis holotoxin remain to be elucidated. An increasing number of other proteins secreted and exported by gram-negative bacteria have been shown to require the gene products of adjacent loci (16, 17). In many instances these gene products have been shown to be required for and specifically dedicated to the efficient processing, assembly, and export of such proteins. Using three independent approaches, we and others (3, 28) have identified a region located adjacent to the 3′ end of the *ptx* operon which is specifically required for the export of pertussis holotoxin. In this report we provide evidence that this region (referred to as the *ptl* operon [28]) is also required for efficient posttranscriptional expression of pertussis holotoxin and that the *ptl* export genes are regulated at the transcriptional level from the *ptx* promoter.

MATERIALS AND METHODS

Strains, plasmids, and media. Brief descriptions of the strains and plasmids used in this study are presented in Table 1. *B. pertussis* strains were grown on solid Bordet-Gengou medium (Difco Laboratories, Detroit, Mich.) containing 15% defibrinated horse blood (Crane Biologics, Syracuse, N.Y.) or in modified Stainer-Scholte liquid medium containing 0.1% 2,6-O-methyl-b-cyclodextrin (Teijin Limited, Tokyo, Japan) as described previously (4). Cultures grown under modulating conditions contained 50 mM $MgSO_4$ in both solid and liquid media. When antibiotics were included in either type of medium, they were added to the following concentrations: ampicillin, 50 μg/ml; kanamycin, 25 μg/ml; streptomycin, 200 μg/ml; and tetracycline, 10 μg/ml. Growth of the liquid cultures was monitored by measuring the optical density at 650 nm (OD_{650}). Mid- to late-logarithmic-phase cultures (OD_{650}, 1.0 to 1.5) were used for most assays. *E. coli* strains were grown in Luria-Bertani medium (19), with ampicillin added to 100 μg/ml when required.

Plasmid constructions and genetic manipulations. Restriction enzymes, T4 DNA ligase, T3 or T7 RNA polymerase, and other enzymes and nucleotides used for DNA or RNA manipulation were obtained from Boehringer Mannheim (Indianapolis, Ind.), Bethesda Research Laboratories Inc. (Bethesda, Md.), or New England Biolabs (Beverly, Mass.). DNA sequencing was carried out with model 373A DNA sequencer (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. Computer searches of the Entrez database (National Institutes of Health, Bethesda, Md.) were done on a Macintosh computer with MacVector software (International Biotechnologies Inc., New Haven,

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TABLE 1. Descriptions of bacterial strains and plasmids used in this study

B. pertussis strain or plasmid	Description
Strains	
PBCC526K	*ptxΔ1*::Kan^{r}Str^s Str^r (Kan^r Str^s marker replacing *ptx* 5′ region with unlinked Str^r allele, used for allelic exchange of *ptxS1* mutants)
PBCC537	*ptxΔ4*::Kan^r (Kan^r replacing entire 4.6-kb *Eco*RI-*Bam*HI *ptx* region)
PBCC548	PBCC526K containing *fhaB*::Tet^r
PBCC554	*ptxS1 R9K W26I* Str^r *fhaB*::Tet^r (*fhaB*$^-$ derivative containing double mutation in S1)
PBCC556	Str^r *fhaB*::Tet^r P_{tac}-*ptx* (*E. coli tac* promoter fused to *ptx*)
PBCC557	PBCC554 containing *ptlG*::Kan^r
PBCC558	PBCC554 containing Δ*ptlC*::Kan^r
PBCC561	PBCC558 with integrated Amp^r pPX2833
PBCC562	Isogenic to PBCC548 but derived from PBCC554
PBCC563	PBCC558 with Δ*ptlC*::Kan^r replaced with wild-type sequences
PBCC577	PBCC554 containing 3′ *ptlC*::Kan^r
Plasmids	
pPX2557	Genomic clone in pUC18 containing 8 kb of 3′ *ptx*
pPX2579	2-kb *Sal*I *ptlF ptlG* subclone containing *ptlG*::Kan^r
pPX2817	−167 to +327 from transcription start of *ptx* in pT3-T7
pPX2833	3.7-kb *Bgl*II subclone containing *ptlC* in pUC18
pPX2834	Δ*ptlC*::Kan^r (Kan^r insert in *ptlC* of pPX2833)
pPX2856	4.5-kb *Bam*HI-*Sal*I subclone containing 3′ *ptlC*::Kan^r
pPX2857	3.7-kb *Bgl*II *ptlC* fragment in chromosomal replacement vector
pPX2862	−361 to −531 from translation start of *ptlC* in pT3-T7
pPX2882	131 bp of *ptlG* and 33 bp of *ptlH* coding region in pSELECT
pPX2894	609 bp of *ptlD* coding region in pSELECT

Conn.). Plasmid constructions and other DNA manipulations were performed under standard conditions (19).

Plasmid pPX2557 (Table 1 and Fig. 1B) was obtained by selecting Kan^r recombinants in pUC19 from an *Eco*RV genomic digest of a *B. pertussis* strain (similar to PBCC526K; Fig. 1) which contains a Kan^r marker replacing the 5′ region (*Kpn*I to *Sal*I) of the *ptx* operon. The resulting pPX2557 plasmid contains 2.7 kb of the *ptx* operon and ~8 kb of the adjacent 3′ region. A 3.7-kb *Bgl*II fragment containing most of *ptlC* was subcloned from pPX2557 into the *Bam*HI site of pUC18 to create pPX2833 (Table 1). The *ptlC* gene in pPX2833 was disrupted by digestion with *Bam*HI and insertion of the Kan^r marker (from pUC4K; Pharmacia LKB, Piscataway, N.J.), which replaces 438 bp of the *ptlC* coding sequence in the resulting plasmid, pPX2834 (Table 1, Fig. 1B). Plasmid pPX2857 (Table 1) was constructed by ligating the same 3.7-kb *Bgl*II fragment from pPX2833 into a chromosomal replacement vector similar to the RTP vector described previously (24). The 3′ coding region of *ptlC* (contained in the 4.5-kb *Bam*HI-*Sal*I fragment) was interrupted by ligating the Kan^r marker into the unique *Bgl*II site to yield pPX2856 (Table 1, Fig. 1B). Disruption of the *ptlG* open reading frame (ORF), contained in a 2-kb *Sal*I fragment, was achieved by insertion of a Kan^r marker into the unique *Cla*I site to create pPX2579 (Table 1, Fig. 1B).

Plasmids were introduced into *B. pertussis* by electroporation with a Transfector 100 electroporator (BTX, San Diego, Calif.) equipped with a 0.5-mm electrode and a Power Plus module. Cells were prepared for electroporation by harvesting mid-logarithmic-phase cultures (OD_{650}, 0.8 to 1.0), washing them in one fourth the culture volume of 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.2, and resuspending them in a 1/50 volume of 1 mM HEPES, pH 7.2, containing 10% glycerol, prior to storage at −70°C (29). During electroporation, the amplitude was set to obtain a pulse of 28 to 30 kV/cm. Cells were preincubated in liquid medium for 60 min at 37°C and plated onto selective medium. For gene disruption experiments, pUC-based vectors (which do not replicate in *B. pertussis*) were used and selection for the Kan^r marker used to replace or interrupt the gene of interest was performed. Double recombination events (between the regions of the gene flanking both sides of the marker and the homologous chromosomal sequences) were scored by determining sensitivity to ampicillin. Plasmids derived from the pPX2777 vector were used to replace chromosomal sequences by first streaking the Amp^r transformants onto streptomycin plates and then screening for the appropriate phenotype as described previously (24).

RNase protection. RNA antisense probes were prepared from clones contained in pT3-T7 (Boehringer Mannheim) or pSELECT-1 (Promega, Madison, Wis.) by transcription of the linearized templates in the presence of [α-^{32}P]GTP (Amersham, Arlington Heights, Ill.). Total cellular RNA was isolated from mid-logarithmic-phase cells by a modification of the hot-phenol method (26). RNA (50 μg) was incubated with 10^5 cpm of the radiolabeled probe in a final volume of 30 μl of hybridization buffer at 95°C for 3 min prior to hybridization overnight at 58°C. Single-stranded RNA was digested with RNase A and RNase T1 (Boehringer Mannheim) as described elsewhere (5). The protected RNA was fractionated on a denaturing 8% polyacrylamide gel along with *Msp*I-digested pBR322 radiolabeled size standards.

Immunochemical assays. Colony immunoblots of *B. pertussis* were performed by standard procedures (19) after growing cells on nitrocellulose filters (BA85; Schleicher & Schuell, Keene, N.H.) for 2 to 3 days. PT was detected with a goat anti-PT antibody fraction and horseradish peroxidase-conjugated rabbit anti-goat antibody (Zymed, San Francisco, Calif.). Positive colonies were visualized by incubation with 4-chloro-1-naphthol and hydrogen peroxide. PT was adsorbed from culture supernatants onto fetuin agarose (Sigma Chemical Co., St. Louis, Mo.) as previously described (4). The adsorbed PT was analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) (6) with SDS–16% polyacrylamide gels. For Western blots (immunoblots) whole cell lysates were resolved on SDS–16% polyacrylamide gels, electroblotted onto nitrocellulose (BA85; Schleicher & Schuell), and probed with goat anti-PT and horseradish peroxidase-conjugated rabbit anti-goat antibody as described above or probed with a mixture of monoclonal antibodies to S1, S2, and S4 (20, 21) and alkaline phosphatase-conjugated goat F(ab′)$_2$ anti-mouse antibodies (Tago Inc., Burlingame, Calif.). The alkaline phosphatase conjugates were developed with 4-nitro blue tetrazolium chloride (Sigma) and 5-bromo-4-chloro-2-indolylphosphate

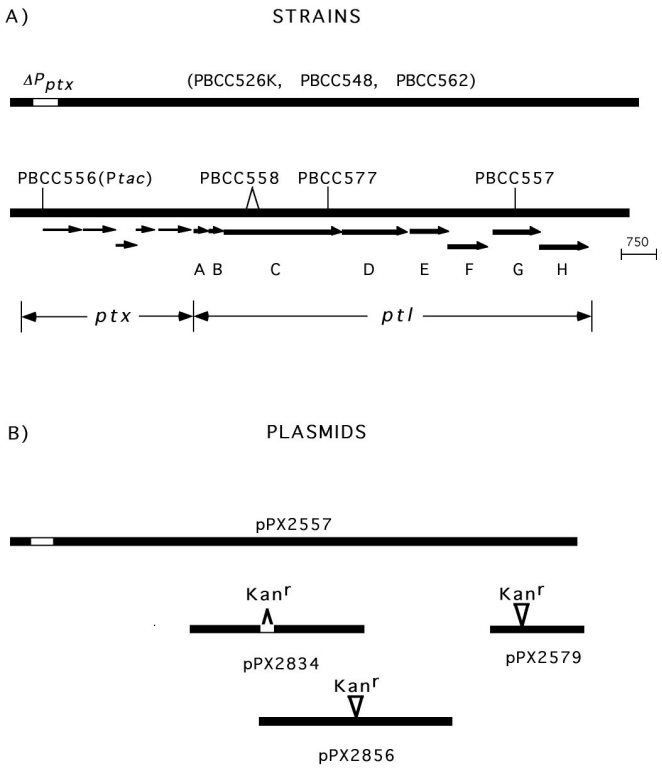

FIG. 1. (A) The top solid line represents the chromosomal region containing the *ptx* and *ptl* loci, with the ΔP_{ptx} region of the corresponding strains designated by the open area. The second solid line depicts the locations of the mutations present in the different strains with respect to the ORFs (depicted by arrows and labeled A to H for the *ptl* region). (B) Chromosomal regions of cloned plasmid vectors used for disruption of different regions of *ptl*. The Kan^r insert of pPX2557 is represented by the open box. Subclones of pPX2557 used for disruption of different regions of *ptlC* (pPX2834 and pPX2579) and *ptlG* (pPX2856) are shown below. The sites of insertion of the Kan^r marker in the various subclones are shown and correspond to the location of the chromosomal mutation depicted in panel A.

toluidinium (Sigma). Volumes of samples used for SDS-PAGE and Western blots were normalized to the culture OD. Hemagglutination assays were conducted as previously described (22), with a 1% suspension of washed goose erythrocytes (Crane Biologics).

The level of toxin in the culture supernatant and in the periplasmic space was determined by an antigen-capture enzyme-linked immunosorbent assay (ELISA). The integrity of the toxin was assessed by ELISA-based assays in which the binding ability of the toxin to either fetuin or the PT receptor in Chinese hamster (CHO) cells was measured. Microtiter plates were coated with either goat polyclonal antibodies to PT for the antigen-capture ELISA, fetuin for the fetuin-binding ELISA, or CHO cell cytoplasmic membranes for the CHO membrane-binding ELISA. The CHO cell membranes were isolated (2) from CHO-K1 or CHO-15B cells. Since the receptors in the ricin-resistant CHO-15B cells are defective and can no longer bind PT (7), the difference between the extent of PT binding to the CHO-K1 membranes and that to the CHO-15B membranes was taken to represent the specific binding of PT to the receptor.

Unless otherwise specified, the following steps for ELISAs were performed at 37°C for 1 h. Coated plates were blocked with 2% bovine serum albumin. The culture supernatant as well as the periplasmic fraction was serially diluted and added to the plates for incubation. The bound toxin was probed with a mouse polyclonal serum to PT followed by alkaline phosphatase-conjugated goat $F(ab')_2$ anti-mouse immunoglobulins (Tago Inc.). The plates were then developed with 1 mg of *p*-nitrophenyl phosphate per ml for 30 min at room temperature, and the OD_{410} was measured.

In each ELISA, purified PT was used to establish a standard curve. The specific binding activity of PT, defined as OD/amount of protein (in micrograms), is derived from the linear portion of a plot of net OD versus PT concentration. On the basis of these standard curves, the toxin concentrations of the test samples were calculated. The detection range is from 1 to 10 ng/ml for the antigen-capture ELISA, from 8 to 80 ng/ml for the fetuin-binding ELISA, and from 3 to 30 ng/ml for the CHO-membrane-binding ELISA.

The periplasmic fractions used in the above ELISAs were prepared with polymixin B (Sigma) treatment of concentrated whole cells (14). The level of toxin recovered in these fractions was normalized to the relative yield of the periplasmic material as estimated by the β-lactamase activity present in the periplasmic and cellular fractions of PBCC561 and other Amp^r control strains.

RESULTS

In the process of examining *B. pertussis ptx* deletion host strains (e.g., PBCC537 [Table 1]) for the expression of PT in *trans* from an intact 4.6-kb *Eco*RI-*Bam*HI plasmid copy of *ptx*, it was discovered that strains containing a corresponding 4.6-kb *ptx* chromosomal deletion were unable to export PT as determined by fetuin-binding ELISA. The lack of PT in the culture supernatant occurred despite the fact that all of the isolates tested were positive for other *bvg*-dependent functions: colonies remained hemolytic and were positive for PT on colony immunoblots, liquid cultures produced filamentous hemagglutinin (determined by hemagglutination titers and Western blot) and P.69 pertactin (as determined by Western blot). The inability to export holotoxin into the culture supernatant is not due to a defect in the plasmid or the plasmid-borne *ptx* gene, since holotoxin can be recovered from culture supernatants when the same plasmid is used to transform other *B. pertussis* host strains containing only partial deletions of *ptx* (1a).

Computer analysis of the DNA sequence deleted in PBCC537 reveals an incomplete ORF that starts 697 bp downstream from the end of the *ptxS3* coding sequence. As observed by others (28), the predicted product of this ORF is homologous to the *virB4* gene product, which is thought to be part of a multiprotein complex involved in the transport of the Ti plasmid DNA protein complex across the cell envelope of *Agrobacter tumefaciens* (27). This ORF has been termed *ptlC* (28).

To more clearly establish the involvement of this region in the expression of pertussis holotoxin, we subcloned a portion of the *ptlC* region as a 3.7-kb *Bgl*II DNA fragment and then replaced an internal *Bam*HI fragment of *ptlC* with a marker for kanamycin resistance (see Materials and Methods). The disrupted *ptlC* gene contained on pPX2834 (Table 1 and Fig. 1B) was used to replace the intact chromosomal copy of *ptlC* by electroporation of PBCC554 (Table 1). All of the hemolytic, Kan^r Amp^s colonies tested positive for PT by colony immunoblot. Twelve isolates were further screened for secretion of PT by testing the culture supernatants for hemagglutination activity. Since the filamentous hemagglutinin gene in PBCC554 is disrupted by insertion of a Tet^r marker (Table 1), hemagglutination activity is a direct measure of the presence of PT. All 12 isolates tested were negative for any hemagglutination activity. One of these, PBCC558, was used for further characterization.

Initial attempts to restore toxin assembly or export in the PBCC558 mutant by direct integration of the uninterrupted portion of the *ptlC* reading frame contained as part of pPX2833 (see Materials and Methods) were unsuccessful, as 15 of 18 hemolytic, Amp^r transformants that expressed PT on colony immunoblots had only marginal hemagglutination activity (titers of 1:2 to 1:4) in the culture supernatants. One of these was saved as PBCC561 (Table 1). To better demonstrate that the defect present in PBCC558 could be completely restored by DNA sequences encoding PtlC, the 3.7-kb *ptlC Bgl*II fragment from pPX2833 was subcloned into a chromosomal replacement vector. The resulting plasmid, pPX2857 (Table 1), facilitates the replacement of the disrupted chromosomal region of PBCC558 without integration of vector sequences (see Materials and Methods). Two isolates of the appropriate phenotype (Amp^s Kan^s Str^r and hemolytic) were obtained, and both contained levels of hemagglutination activity that were identical to that of the PBCC554 parental strain. One of the isolates was saved as PBCC563 (Table 1) for further characterization.

The failure of any of the pPX2833 transformants to restore the PT hemagglutination-negative phenotype of PBCC558 prompted us to investigate the possibility of additional downstream genes involved in the export of PT. Putative coding regions downstream of the PBCC554 *ptx* chromosomal gene were interrupted by allelic exchange by electroporating PBCC554 with pPX2856 and pPX2579 (Table 1). The Amp^s Kan^r hemolytic isolates obtained with either plasmid had phenotypes similar to that of PBCC558 in that, of the ~90% that remained positive for PT on colony immunoblots, all were negative for hemagglutination. Two typical isolates were saved as PBCC577 and PBCC557. Subsequent DNA sequencing of the region cloned in pPX2557 reveals eight ORFs which correspond to the *ptlA-ptlH* genes described by Weiss et al. (28). Accordingly, PBCC577 contains the Kan^r gene inserted in the 3′ region of *ptlC* and PBCC557 contains a Kan^r insertion in the middle of *ptlG* (Fig. 1).

To more precisely distinguish the involvement of PtlC in the synthesis, secretion, assembly, or export of holotoxin, the distribution of holotoxin and subunits was examined in the *ptlC* mutant and wild-type strains. The results of SDS-PAGE (Fig. 2A) show that, compared with those from the *ptlC* wild-type strain, culture supernatants obtained from the *ptlC* mutant strains (PBCC558 and PBCC561) are depleted for extracellular PT in a form capable of binding fetuin (which requires an intact B oligomer) and that no detectable PT is recovered from the Ptx^- negative control, PBCC562. Western blot of the fetuin-adsorbed fractions from the above-mentioned strains (Fig. 2B) shows that the S1 subunit (upper band) is readily detected in PBCC554 and PBCC563 and is present at lower levels in the two *ptlC* mutant strains PBCC558 and PBCC561. The presence of S1 in the fetuin-adsorbed fractions from the two *ptlC* mutant strains indicates that a low level of holotoxin (as opposed to only the B oligomer) is exported. The presence of wild-type levels of PT (Fig. 2A) and S1 (Fig. 2B) recovered from PBCC563 also shows that repair of the disrupted *ptlC* restores the capacity to export holotoxin. The faint band detected in the PBCC562 negative control (Fig. 2B) is most likely due to a low

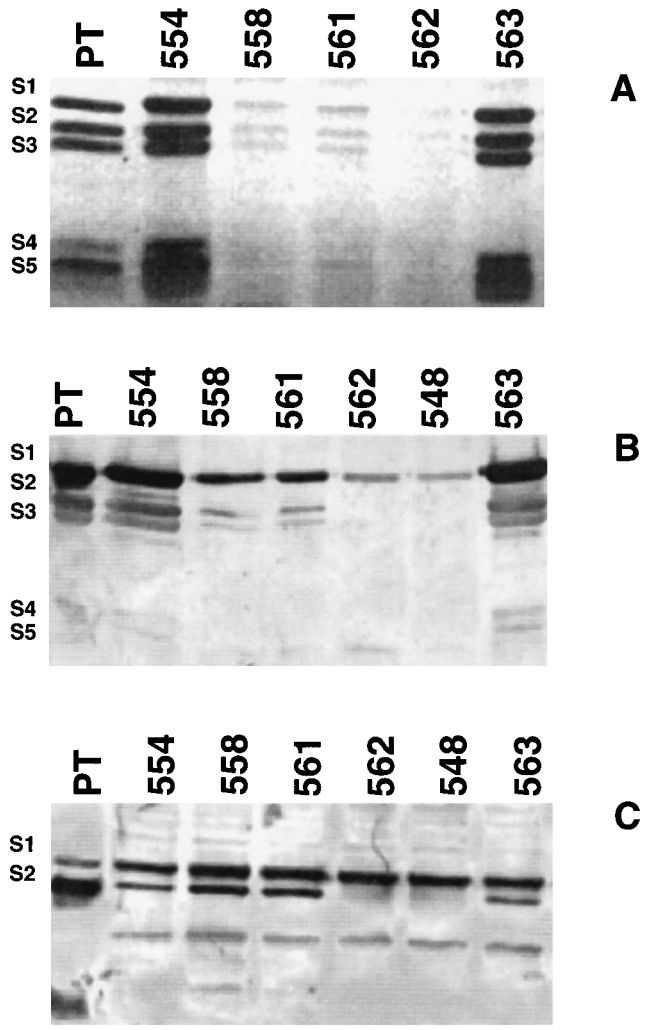

FIG. 2. (A) The presence of PT in culture supernatants from the indicated strains was determined by SDS-PAGE of the fetuin-adsorbed protein as described in Materials and Methods. S1, S2, S3, S4, and S5 indicate the positions of the respective PT subunits. Lane PT contains 5 μg of purified PT. (B) Western analysis of fetuin-adsorbed fractions from the indicated strains with polyclonal anti-PT. Lanes 558, 561, 562, and 548 (indicating the respective PBCC strains) contain twice the amount of material present in lanes marked 554 and 563. Lane PT contains 2 μg of purified PT. (C) Western analysis of whole cell lysates of the indicated strains with a mixture of monoclonal antibodies as described in Materials and Methods. Images were labeled and density adjustments were made on a Macintosh Centris 650 computer, with the Canvas and Adobe Photoshop programs, respectively.

level of a cross-reactive protein that migrates with or slightly below the S1 protein. This protein is detected by the polyclonal antisera and is also present in another *ptx* deletion strain, PBCC548 (Fig. 2B). When a mixture of monoclonal antibodies (20, 21) specific for the S1, S2, and S4 subunits is used, Western blots of whole cell lysates (Fig. 2C) show that the S2 subunit is detected in lysates of all strains except the two Ptx^- strains, PBCC562 and PBCC548. The S4 subunit is not reproducibly detected in these lysates, and detection of the S1 subunit is again partially obscured by the presence of the cross-reacting protein. Similar experiments have shown that this cross-reacting band (also reported by Covacci and Rappuoli [3]) is present in all *B. pertussis* strains examined, whether grown under modulating or nonmodulating conditions (results not shown).

We have developed a set of three different ELISAs that permit the estimation of the amount of PT protein present (antigen-capture ELISA) relative to the amount of functional PT (B oligomer or holotoxin) capable of binding to either fetuin-coated plates or CHO cell membrane-coated plates (see Materials and Methods). These three ELISAs were used to determine whether the defect present in PBCC558 is due to the failure of the strain to assemble the toxin subunits into a form that can bind fetuin or CHO cell membranes or whether the defect is due to an impaired capacity to export the assembled holotoxin from the periplasm. In the particular experiment shown (Table 2), the CHO membrane- and fetuin-binding ELISAs provide a slight overestimation (1.3- and 1.4-fold, respectively) of the amount of PT detected in the PBCC554 (wild-type) supernatant compared with the estimate obtained with the antigen-capture ELISA (96.4 μg). Despite the slight discrepancy in these control values, all three assays indicate that less than ~3% of the total PT protein (extracellular plus periplasmic) is present in the periplasm of the wild-type strain. In contrast, the total amount of PT in the *ptlC* mutant PBCC558 is severely reduced, and this reduction can be attributed almost entirely to the lack of extracellular PT (Table 2). The small amount (3.6 μg, or ~4% of wild-type level) of extracellular PT that is detected by the antigen-capture ELISA of PBCC558 culture supernatants also appears functional in the fetuin- and CHO cell membrane-binding ELISAs (~80 to 90% of the extracellular PT detected by antigen binding is also active in the CHO and fetuin-binding assays [Table 2]). The antigen-binding assay shows that the amount of periplasmic PT detected in the PBCC558 mutant is very similar to the PBCC554 wild-type level (2.8 and 3.3 μg, respectively). The CHO membrane- and fetuin-binding assays also detect similar amounts of periplasmic PT (1.1 to 1.4 μg) in the mutant and wild-type strains. In both *ptlC* mutant and wild-type strains, approximately the same proportion (40 to 50%) of the periplasmic PT detected by antigen binding is active in the fetuin- and CHO membrane-binding assays. These results (Table 2) also show that, compared with the PBCC558 parental strain, the integrated copy of pPX2833 present in PBBCC561 does not result in any significant increase in the amount of periplasmic or extracellular PT. In all three ELISAs, the PBCC562 negative control lacks any detectable PT in either the periplasmic or extracellular fraction. The results of separate studies (data not shown) demonstrate that restoration of *pltC* to the wild-type configuration (PBCC563) restores wild-type levels of extracellular PT as determined by the three different ELISAs.

To determine if the *ptlC* mutation alters the transcription of *ptx*, RNA was hybridized to an RNA antisense probe (pPX2817 [Table 1]) overlapping the −167-to-+327 region of the *ptx* transcription initiation site. These results (Fig. 3) show that the RNA from the Tohama strain (Fig. 3, lane 4) protects a region of the probe (corresponding to approximately 327 nucleotides [nt]) expected if transcription initiates at the published site (13). RNA from PBCC554 (Fig. 3, lane 5) protects two regions of the probe, corresponding to ~150 and ~120 nt. The 150-nt band is expected if transcription of *ptx* starts at the same transcription initiation site and extends through the *ptxS1 R9K* mutation present in PBCC554 (Table 1). The 120-nt band is the expected digestion product corresponding to the distance from the *W26I* mutation in PBCC554 (Table 1) to the end of the probe. For each of the mutations, the 3-bp mismatch of the RNA duplex formed by hybridization of the wild-type sequence of the probe with mRNA corresponding to the mutated regions in PBCC554 (and its derivatives) results in digestion by RNase to yield the observed pattern. RNAs from PBCC558

TABLE 2. Comparisons of PT in culture supernatants and periplasmic fractions from *B. pertussis* strains containing mutations in *ptlC*

Strain	OD_{650}	Fraction[a]	Amt (μg) of PT determined by ELISA[b]		
			Antigen capture	Fetuin binding[c]	CHO binding[c]
PBCC554	1.52	Supernatant	96.4	125 (1.3×)	135 (1.4×)
		Periplasm	3.3	1.3 (0.4×)	1.3 (0.4×)
PBCC558 (*ptlC*)	1.30	Supernatant	3.6	2.9 (0.8×)	3.2 (0.9×)
		Periplasm	2.8	1.4 (0.5×)	1.1 (0.4×)
PBCC561 (*ptlC*)	1.19	Supernatant	2.8	2.8 (1.0×)	2.2 (0.8×)
		Periplasm	3.6	1.1 (0.3×)	1.1 (0.3×)
PBCC562 (Δ*ptx*)	1.46	Supernatant	<0.001	<0.008	<0.003
		Periplasm	<0.001	<0.008	<0.003

[a] Values listed for the periplasmic fractions from each strain are normalized to the percentage of the fraction recovered.
[b] Values listed represent micrograms of PT recovered from 50-ml cultures. The values listed for PBCC562 are below the indicated limits of detection (1 μg/ml of culture) of the respective assays.
[c] Values listed in parentheses are fractions or multiples of the amounts of PT detected by the antigen-capture ELISA.

(Fig. 3, lane 6), PBCC561 (lane 7), and PBCC563 (lane 9) also protect the same regions of the probe from RNase digestion. As predicted, PBCC562 (lane 8) lacks any detectable *ptx* mRNA.

Initial efforts to identify the transcription start site for *ptlC* utilized an antisense RNA probe complementary to a region 89 bp before the predicted translation start site and including 370 bp of the complementary coding sequence. Using this probe for RNase protection of the mRNA synthesized by the wild-type (Tohama) strain grown under nonmodulating conditions revealed that the entire length of the probe was protected from RNase digestion (data not shown), indicating that the transcription start site for *ptlC* occurs upstream of the region probed. An RNA antisense probe (pPX2862) extending further upstream (within the putative *ptlA* gene, or −361 to −531 bp from the predicted translation start of *ptlC*) was used to hybridize to RNAs from the wild-type strain grown under both modulating and nonmodulating conditions. The results of this experiment show that a 169-nt region of the probe is protected only when used to hybridize to RNA from the wild-type strain grown under nonmodulating conditions (Fig. 4, lane 3). Although the RNase-treated and untreated probe controls (lanes 2 and 5, respectively) reveal incomplete digestion of the probe, it is clear that the 169-nt protected band is not detected when the probe is hybridized to RNA from the modulated wild-type strain (lane 4). The size of this 169-nt protected band is that expected if the entire length of the probe, minus ~60 nt corresponding to vector sequences, is protected from RNase digestion. Additional RNA mapping experiments (data not shown) have not identified the 5′ end of the *ptl* mRNA within the 696-bp region between the *ptlC* and *ptxS3* coding sequences.

The inability to identify the site of initiation for *ptl* transcription prompted us to examine the transcription of *ptx* and *ptl* genes in a wild-type strain, a strain containing a promoter

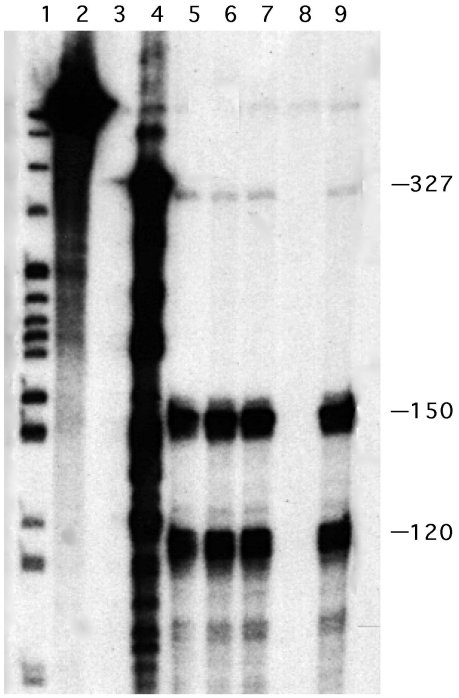

FIG. 3. RNA from strains hybridized to an RNA probe overlapping the *ptx* transcription start. Lane 1, DNA size standards; lane 2, undigested probe; lane 3, digested probe; lane 4, RNA from Tohama wild type (with the major protected band appearing at ~327 upon lower exposure); lane 5, PBCC554 (*ptxS1 R9K W26I*); lanes 6 to 9, PBCC558, PBCC561, PBCC562, and PBCC563, respectively (derivatives of PBCC554). The approximate size and location of the two bands (150 and 120 nt) produced from the *ptxS1 R9K W26I* allele is shown on the right. The image was labeled and reproduced as described in the legend to Fig. 2.

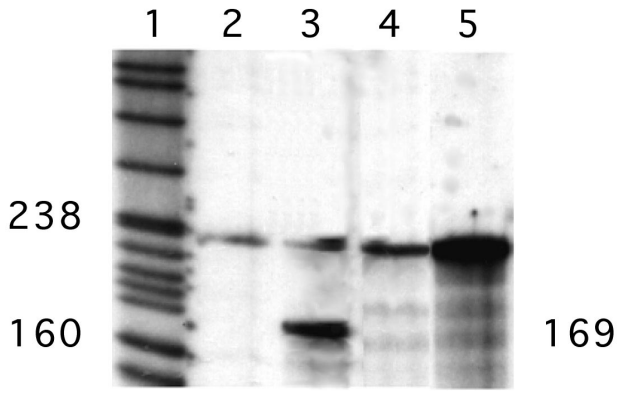

FIG. 4. RNase protection assay with a probe extending from −526 to −361 from the *ptlC* translation start. Lane 1, DNA size markers, with relevant positions indicated; lane 2, digested probe control; lane 3, RNA from Tohama wild type grown without $MgSO_4$; lane 4, RNA from Tohama wild type grown in the presence of $MgSO_4$; lane 5, undigested probe. The image was labeled and reproduced as described in the legend to Fig. 2.

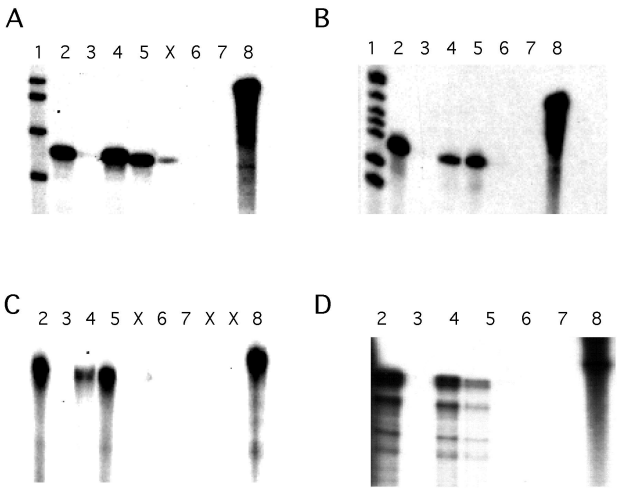

FIG. 5. (A) 5′ *ptx* mRNA probe (−167 to +327 from transcription start). The undigested 544-nt probe in lane 8 routinely migrates more slowly than expected relative to the DNA size standards. The band present in lane X is due to a small amount of material inadvertently introduced from lane 5. (B) *ptlA* probe (−361 to −531 from translation start of *ptlC*). (C) *ptlD* probe (609 bp internal to *ptlD* coding region). (D) *ptlG* and *ptlH* probe (131 bp of 3′ coding region of *ptlG* and 33 bp of 5′ coding region of *ptlH*). The images were labeled and reproduced as described in the legend to Fig. 2. Lanes for all panels: 1, pBR322 *Msp*I size standard; 2, wild type (Tohama); 3, wild type grown in $MgSO_4$; 4, P_{tac}-*ptx* strain PBCC556; 5, P_{tac}-*ptx* strain PBCC556 grown in $MgSO_4$; 6, *ptx*Δ*1* strain PBCC526K; 7, no RNA (digested probe); 8, no RNase (undigested probe); X, blank lane.

deletion, ΔP_{ptx}, (PBCC526K, [Table 1]), and a strain in which P_{ptx} has been replaced by the *E. coli tac* promoter (PBCC556 [Fig. 1 and Table 1]). When the same *ptx* probe described above (−167 to +327 in pPX2817) is used, the RNase protection assay shows that the 327-nt protected band is not detected in the wild type grown under modulating conditions (Fig. 5A, lane 3) or in the ΔP_{ptx} PBCC526K strain (Fig. 5A, lane 6). The protected band is present in the wild type grown under nonmodulating conditions (Fig. 5A, lane 2) and in PBCC556 grown under both conditions (Fig. 5A, lanes 4 and 5). The presence of *ptx* mRNA in PBCC556, despite the presence of $MgSO_4$, supports previous work (16) which has shown that PBCC556 and similar P_{tac} promoter substitution strains can synthesize and export wild-type levels of pertussis holotoxin during growth in either the presence or absence of $MgSO_4$.

Hybridization of the pPX2862 *ptlA* antisense probe to the same set of strains shows that only RNA prepared from the wild type grown in the absence of $MgSO_4$ (Fig. 5B, lane 2) or RNA prepared from PBCC556 (grown with or without $MgSO_4$ [Fig. 5B, lanes 5 and 4, respectively]) can protect a 169-nt region of the probe from digestion. Transcription of *ptlA* is not detected in the modulated wild-type (Fig. 5B, lane 3) or ΔP_{ptx} strain, PBCC526K (Fig. 5B, lane 6). Hybridization of a 609-nt probe (pPX2894) derived from within the *ptlD* coding region also results in protection of the full-length probe (minus the polylinker region) only in the unmodulated Tohama strain (Fig. 5C, lane 2) and in PBCC556 (grown with or without $MgSO_4$ [Fig. 5C, lanes 5 and 4, respectively]). The lack of any detectable band in the modulated wild-type strain (Fig. 5C, lane 3) and the PBCC526K *ptx* promoter deletion strains indicates that transcription of *ptlD* is also subject to modulation by $MgSO_4$ and that *ptlD* is not transcribed in the absence of a functional *ptx* promoter. Finally, hybridization to an antisense probe (pPX2882) derived from the 3′ coding region of *ptlG* (131 bp) and the 5′ coding region of *ptlH* (33 bp) results in a similar pattern of protection. RNAs from the unmodulated Tohama strain (Fig. 5D, lane 2) and modulated or unmodulated PBCC556 (Fig. 5D, lanes 5 and 4, respectively) protect an ~161-nt region of the probe, indicating that the entire *ptlG* or *ptlH* region of the probe is protected.

DISCUSSION

We have shown that at least one of the *ptl* genes (*ptlC*) in *B. pertussis* is involved in the export of holotoxin. Disruption of *ptlC* results in a severe depletion of PT in the culture supernatant of the mutant strain; however, the relative proportion of the total periplasmic PT protein that is assembled as holotoxin by the *ptlC* mutant does not differ from those of wild-type strains. These results confirm the previous observation by Covacci and Rappuoli (3) and indicate that the defect conferred by the *ptlC* mutation is not a consequence of defective assembly of PT but is due to the inability of the mutant strain to export the assembled pertussis holotoxin from the periplasm across the outer membrane. These results also indicate that the assembly of holotoxin in *B. pertussis* occurs independently of the export process.

The approximately 30-fold reduction in the level of extracellular PT synthesized by the *ptlC* mutant is the consequence of posttranscriptional regulation, since the steady-state level of *ptx* mRNA is not affected by the *ptlC* mutation. Although we have not excluded the possibility that the overall reduction of PT is due to increased degradation of the individual subunits, we have been unable to detect any increase in degradation products either in the periplasm (Table 1) or in whole cell lysates (Fig. 3C). In contrast, whole cell lysates of the *ptlC* mutants appear to contain slightly elevated levels of intact S1 and S2 PT subunits detected by Western blot (e.g., PBCC558 and PBCC561 [Fig. 2C]). Whole cell lysates of another *ptlC* mutant (BP3171) have been reported to contain elevated levels of PT activity (28). Since the results of this study show that the *ptlC* mutants do not contain elevated levels of periplasmic PT, it appears that impaired export of holotoxin in these mutants leads to slightly increased cytoplasmic (or membrane-associated) steady-state levels of one or more of the subunits. At present we cannot distinguish whether the apparent accumulation of the PT subunits causes feedback inhibition of *ptx* mRNA translation or whether reduced translation is a more direct effect of a lesion in the export pathway, as observed in other cotranslational translocation systems (15).

The *ptl* operon is subject to virulence modulation and is regulated at the transcriptional level in a manner similar to those of the other *bvg*-regulated genes in *B. pertussis*. Initiation of transcription of the *ptl* operon cannot be detected in the ~700-bp region between the 3′ end of the *ptx* S3 coding region and the beginning of the *ptlC* coding region. At least for the *ptl* regions tested, *ptl* is not transcribed under modulating growth conditions or in strains lacking the *ptx* promoter. When transcription of *ptx* is uncoupled from *bvg* regulation by fusion to P_{tac} (as in PBCC556), expression of PT is independent of *bvg* and holotoxin is recovered in culture supernatants, even when $MgSO_4$ is included in the growth medium (3a). The results showing that RNA from $MgSO_4$-modulated PBCC556 protects all three *ptl* probes (Fig. 5B to D) demonstrate that the corresponding export genes are transcribed under these conditions. These results also indicate that the effects of modulation of PT and export gene expression are primarily limited to regulation at the transcriptional level, since similar levels of holotoxin are recovered under either growth condition. Taken together, these results strongly suggest that the *ptl* operon is transcribed from the *ptx* promoter as part of a long (>11-kb) polycistronic mRNA. The inability to detect the entire *ptx-ptl*

mRNA by Northern (RNA) hybridization (data not shown) is likely to reflect the lability of the intact (>11-kb) transcript during RNA isolation. Although mRNA breakdown or premature termination products can sometimes be detected by RNase mapping (especially in the more distal genes [e.g., the lower bands in Fig. 5D]), transcription of the *ptx-ptl* operon is remarkably processive and the *ptx* portion of the transcript appears to be stable even under the conditions of reduced translation of *ptx* mRNA in the *ptlC* mutants (Fig. 3, lane 6 and 7). The processive transcription of *ptx-ptl* does not appear to be due the *ptx* promoter since substitution by P_{tac} results in approximately the same level (within experimental error) of transcription of the distal genes.

The conclusion that expression of the *ptl* operon is regulated from the *ptx* promoter has implications for this and previous works. One of these implications concerns conflicting reports of the ability to recover extracellular holotoxin from *trans* expression of *ptx* contained on autonomously replicating plasmids. Depending in part upon the particular combination of *Bordetella* host strain and *ptx*-containing plasmids employed, in several instances (for examples, see reference 7; also reference 1a) it is possible to recover extracellular holotoxin from host strain-plasmid combinations that would not be predicted to express *ptl*. In such combinations, the lack of chromosomal *ptx* promoters in the hosts should preclude the expression of the downstream *ptl* genes (which are not present in *trans* on the *ptx*-containing plasmid). In all of these instances however, the Rec^+ host strain-plasmid combinations share homologous regions which provide potential sites for recombination events to restore the wild-type *ptx-ptl* chromosomal configuration. It is only with those plasmid-Δ*ptx* host chromosome combinations that do not share homologous regions that we have been unable to recover holotoxin from culture supernatants.

The polycistronic nature of the *ptx-ptl* mRNA also increases the likelihood that the phenotypes of the *ptlC* mutants described here (PBCC558, PBCC561, and PBCC577) and elsewhere (3, 28) may be attributed to polar effects upon the remaining *ptl* genes. Such chromosomal insertions of antibiotic resistance genes or entire plasmid vectors might be expected to result in premature termination or increased lability of at least a fraction of the *ptx-ptl* transcripts. Preliminary RNase protection experiments with the PBCC558 *ptlC*::Kan^r mutant (9a) indicate that insertion of the Kan^r gene does reduce the amount of mRNA detected with the distal *ptlD*, *ptlG*, or *ptlH* probes. Although not characterized in the same detail, the export-negative, PT^+ phenotype of PBCC557 (*ptlG*) and of mutations in downstream *ptl* genes (28) is very similar to those of the *ptlC* mutants described here. The common phenotype of the *ptlC* and *ptlG* mutants does not allow distinction between the possible polar effects of the *ptlC* mutations and the epistatic effects of mutations in the downstream genes. Future characterization of nonpolar mutants in different *ptl* genes will provide a more complete understanding of the interactions required for the translocation of macromolecular complexes such as pertussis holotoxin.

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