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Roles of the Disulfide Bond and the Carboxy-Terminal Region of the S1 Subunit in the Assembly and Biosynthesis of Pertussis Toxin

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A *Bordetella pertussis* expression system was developed to analyze the structure-function relationship, in vivo assembly, and biosynthesis of pertussis toxin. The toxin structural gene was first deleted from the *B. pertussis* chromosome; into the resulting *B. pertussis* strain the toxin gene was introduced on a low-copy-number, broad-host-range plasmid. The amount of pertussis toxin produced and secreted with this expression system was in the same order of magnitude as that produced by *B. pertussis* Tohama I, indicating that although the plasmid may be present in more than one copy per cell, overproduction of the toxin was not achieved in *B. pertussis*. Expression of mutant pertussis toxin genes in which the codon for Cys-41 was deleted or altered or in which the carboxy-terminal region was deleted showed that both the single intrachain disulfide bond and the carboxy-terminal region of S1 are essential for the stable expression, assembly, and secretion of S1. On the other hand, the B oligomer was efficiently secreted in the culture medium in the absence of the S1 subunit. The secreted B oligomer contained S2, S3, and S4 subunits as evidenced by enzyme-linked immunosorbent assay and was fully functional with respect to haptoglobin binding. Furthermore, the deletion of the hydrophobic carboxy-terminal region has a drastic effect on S1 subunit solubility; however, inclusion of the hydrophobic region was not sufficient for assembly and secretion, indicating that other interactions involving amino acids beyond residue 207 of the S1 subunit are also required.

Pertussis toxin (PTX) is an exotoxin produced by virulent *Bordetella pertussis*, the causative agent of whooping cough (35). PTX is an oligomeric protein composed of five different subunits, named S1 through S5. The structure of the toxin conforms to the A-B model for bacterial toxins (40). The A protomer is composed of the subunit S1, which expresses ADP ribosyltransferase and NAD glycohydrolase activities (23). The B oligomer is formed of two different dimers, D1 (subunits S2 and S4) and D2 (subunits S3 and S4), held together by the S5 subunit (40). The B oligomer mediates the binding of the holotoxin to the membrane of the eucaryotic target cell and the subsequent translocation of the enzymatically active S1 subunit into the cytoplasm of the cell (41).

Among the different interactions between the five subunits, the interaction of the S1 subunit with the B oligomer is the weakest (40). Release of the S1 subunit is promoted in vitro by the addition of dithiothreitol, ATP or dipolar ionic detergents such as 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) (8, 20, 21, 34). Reduction of the isolated S1 subunit prevents in vitro reassociation of the S1 subunit and the B oligomer (9). In addition, the reduction and most likely the release of the S1 subunit from the B oligomer are required for the expression of both ADP ribosyltransferase and NAD glycohydrolase activities (8, 21, 23, 33). This reduction can be achieved by glutathion in the eucaryotic target cell (21). The gene of PTX has been cloned (27, 36) and sequenced (30, 36). The analysis of the primary structure of the S1 subunit revealed that this protein possesses two cysteine residues at positions 41 and 200. These two cysteines form a disulfide bond in the S1 subunit when the holotoxin is secreted from the bacteria. In vitro modification of the cysteine residues (9, 20, 22) and site-directed mutagenesis (C. Locht, Y. Lobet, C. Feron, W. Cieplak, and J. M. Keith, J. Biol. Chem., in press) of these residues have shown that Cys-41 but not Cys-200 is located near the NAD-binding site of the enzyme.

The amino-terminal portion of the S1 subunit of PTX has amino acid homology to the A subunit of cholera toxin and *Escherichia coli* heat-labile toxin, two ADP-ribosylating subunits acting on substrates similar to that of PTX (30, 36). Furthermore, a truncated form of the PTX S1 subunit produced as a fusion protein in *E. coli*, in which the carboxy-terminal domain was deleted, expressed NAD glycohydrolase activity indistinguishable from that of the entire S1 subunit (29), indicating that the enzyme domain is located in the amino-terminal region of the molecule. The carboxy-terminal portion of the cholera toxin A subunit is involved in the binding of the A subunit with the cholera toxin B oligomer (17). A limited tryptic digestion of the S1 subunit has suggested that the carboxy-terminal part of the molecule is important for the in vitro association of the A protomer with the B oligomer (6), analogous to cholera toxin.

In this study we provide direct evidence that both the disulfide bond and the carboxy-terminal region are essential for the in vivo assembly of the holotoxin and that the B oligomer may be assembled in vivo and secreted in the absence of the S1 subunit.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. *B. pertussis* Tohama I was provided by P. Roelants (Smith Kline-Biologicals, Rixensart, Belgium). *B. pertussis* Tohama I (Sm^r Nal^r) and *B. pertussis* BPRA were developed in this study. *E. coli* TG1 [Δ(*lac-pro*) *supE thi hsdD5*/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15] was purchased from Amersham Corp., Amersham, England. *E. coli* SM10 (38) was provided by S. Stibitz (Food and Drug Administration, Bethesda, Md.). Plasmids pTOX9 (4) and pSS1129 were supplied by W. J. Black (Stanford University, Stanford, Calif.) and S. Stibitz, re-

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TABLE 1. Plasmids used in this study

Plasmid	Characteristics	Source or reference
pLAFRII	Mobilizable, IncP-1 plasmid, Tet^r	13
pTOX9	Mobilizable, ColE1 *oriV*, Amp^r	4
pSS1129	Mobilizable, ColE1 *oriV*, Amp^r Gen^r Sm^s, derivative of pRTP1	39
pUC7	ColE1 *oriV*, Amp^r, multiple cloning site	42
pTXS11	Truncated S1 cistron inserted into pUC18	29
pTXS13	S1 cistron inserted into pUC18	29
pRIT13295	pSS1129 derivative containing the PTX gene-flanking regions	This study
pRIT13294	pLAFRII derivative containing the PTX gene	This study
pRIT13070	pUC7 derivative containing the PTX gene	This study
pRIT13374	Similar to pRIT13070 with deletion of codon 41 in the S1 cistron	This study
pRIT13375	Similar to pRIT13070 with TGC-to-GGC replacement of codon 41 in the S1 cistron	This study
pRIT13376	Similar to pRIT13070 with TGC-to-AGC replacement of codon 41 in the S1 cistron	This study
pRIT13368	pLAFRII derivative containing the PTX gene from pRIT13374	This study
pRIT13369	pLAFRII derivative containing the PTX gene from pRIT13375	This study
pRIT13370	pLAFRII derivative containing the PTX gene from pRIT13376	This study
pRIT13377	Similar to pRIT13070 containing the S1 cistron deletion from codon 187 to codon 234	This study
pRIT13371	pLAFRII derivative containing the PTX gene from pRIT13377	This study
pRIT13378	Similar to pRIT13070 containing the S1 cistron deletion from codon 207 to codon 234	This study
pRIT13380	Similar to pRIT13378 containing a frameshift mutation in the S1 cistron	This study
pRIT13372	pLAFRII derivative containing the PTX gene from pRIT13378	This study
pRIT13373	pLAFRII derivative containing the PTX gene from pRIT13380	This study

spectively. pSS1129 is a derivative of pRTP1 (39), in which a 2.5-kilobase (kb) DNA fragment coding for gentamicin resistance has been inserted. Plasmid pLAFRII (13) was provided by J. J. Mekalanos (Harvard Medical School, Boston, Mass.), and pUC7 (42) was provided by N. Harford (Smith Kline-Biologicals, Rixensart, Belgium). pTXS11 and pTXS13, coding for rS1d and rS1, respectively, were described previously (29). The recombinant phages φRIT20003, φRIT20004, and φRIT20005 were described elsewhere (Locht et al., in press). Briefly, these three phages are M13mp18 derivatives in which the DNA coding for rS1d has been inserted and the codon for Cys-41 has been deleted (φRIT20003) or replaced by the GGC codon for glycine (φRIT20004) or the AGC codon for serine (φRIT20005). All other plasmids were developed in this study. The plasmids used are summarized in Table 1.

E. coli strains were grown in LB broth or minimal medium (31). Antibiotic-resistant *E. coli* strains were selected with 100 μg of ampicillin per ml or 15 μg of tetracycline per ml. *B. pertussis* was grown on Bordet Gengou (BG) agar, or in modified Stainer Scholte (SS) medium containing 1 g of 2,6-*O*-dimethyl-β-cyclodextrin per liter (19) and the relevant antibiotics at the following concentrations: tetracycline, 15 μg/ml; gentamicin, 15 μg/ml; nalidixic acid, 50 μg/ml; and streptomycin, 100 μg/ml. BG agar contained 36 g of BG agar (Difco Laboratories, Detroit, Mich.) per liter, 10 ml of glycerol per liter, and 200 ml of defibrinated sheep blood per liter.

DNA manipulation. Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were purchased from Pharmacia-LKB (Uppsala, Sweden), Boehringer (Mannheim, Germany), or Bethesda Research Laboratories, Inc. (Bethesda, Md.), and used as recommended by the supplier. DNA sequencing was carried out with the T7 sequencing kit from Pharmacia-LKB according to the instructions of the supplier. All other DNA manipulations were performed under standard conditions, as described by Maniatis et al. (31). Southern blot analyses were performed with ^{32}P-labeled DNA probes. The DNA was labeled by nick translation with [α-^{32}P]dCTP (800 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.). The oligonucleotides used for sequencing were synthesized on an automated solid-phase synthesizer and had the following sequences: GGGG ATACCTGCGACGCGCTTGTTCTGGGC (S21), ATAGAC CTCGGTATAGCGCCGGCTG (C1), and TTGATGATGCC GCCGTACGCCTGGCCCAGG (S32). Chromosomal DNA was isolated from *B. pertussis* by the method of Hull et al. (18).

Bacterial conjugation. Freshly grown *E. coli* SM10 cells containing pLAFRII-derived or pSS1129-derived recombinant plasmids were mixed with freshly grown Nal^r Sm^r *B. pertussis* Tohama I or BPRA cells on BG agar plates by using sterile plastic inoculating loops (Nunc InterMed, Roskilde, Denmark). The strains were mated at 36°C for approximately 5 h. The cells were then spread on BG plates containing nalidixic acid and tetracycline, or nalidixic acid and gentamicin, and the plates were incubated for 5 days at 36°C. Antibiotic-resistant hemolytic *B. pertussis* colonies were then grown in liquid SS medium containing the appropriate antibiotics for further analysis.

Analytical procedures. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 12.5% polyacrylamide slab gels was carried out as described by Laemmli (25). Western immunoblot analyses with monoclonal antibodies were performed as described previously (29). The monoclonal antibodies used in this study were described by Francotte et al. (11), Frank and Parker (12), and Marchitto et al. (32). Expression of the recombinant S1 subunit genes in *E. coli* was performed as described earlier (29).

Enzyme-linked immunosorbent assay (ELISA). The wells of polystyrene microdilution plates (4-39454; Nunc) were coated overnight at 4°C with 50 μl of haptoglobin (2 μg/ml, H-1511; Sigma Chemical Co., St. Louis, Mo.) or purified polyclonal rabbit anti-PTX antibodies (2 μg/ml) in 100 mM carbonate buffer (pH 9.5). The plates were washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-Tween). Nonspecific binding sites were blocked with PBS-Tween containing 1% bovine serum albumin and 4% newborn calf serum (saturation buffer) for 6 h at room temperature. The plates were washed again three times with PBS-Tween. PTX (List Biological Laboratories, Inc., Campbell, Calif.) or culture supernatant samples (50 μl) in serial dilutions were then added to each well in PBS-Tween containing 1% bovine serum albumin. After overnight incu-

bation at 4°C, the plates were washed again three times with PBS-Tween. Mouse polyclonal anti-PTX antiserum (1/10,000 dilution) or subunit-specific monoclonal antibodies (5 μg of 22A2 [anti-S1], F6D1 [anti-S2], 59F4 [anti-S3], or 43H10 [anti-S4] per ml) were then added in PBS-Tween containing 1% bovine serum albumin for 2 h at 37°C. After three washes with PBS-Tween, biotinylated anti-mouse immunoglobulin G (RPN.1001; Amersham) was added at a 1/500 dilution for 1.5 h at 37°C in PBS-Tween. The plates were washed again three times with PBS-Tween and then incubated with the streptavidin-biotinylated horseradish peroxidase complex (RPN.1051; Amersham) at a dilution of 1/1,000 for 30 min at 37°C in the saturation buffer. The plates were washed again three times with PBS-Tween and then developed with 0.4 mg of *O*-phenylenediamine dihydrochloride (P-4664; Sigma) per ml and 1 μl of hydrogen peroxide per ml in 0.1 M citrate buffer (pH 4.5) at room temperature in the dark. The plates were read on a Nunc immunoreader NJ2000.

CHO cell cytotoxicity assay. The Chinese hamster ovary (CHO) cell assay was performed essentially as described by Hewlett et al. (15), with minor modifications. Briefly, the CHO cells were grown in RPMI medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2 mM L-glutamine, 50 IU of penicillin per ml, 50 IU of streptomycin per ml, and 10% newborn calf serum at 37°C at 5% CO_2. After trypsinization the cells were suspended in the same medium at 10^5 cells per ml, and 200 μl of this suspension was added to 96-well microdilution plates in the presence of 50 μl of the PTX samples or *B. pertussis* culture supernatants in serial dilutions. After incubation for 48 h at 37°C, the cells were washed with PBS and fixed with methanol. The cells were then Giemsa stained and examined under a light microscope.

RESULTS

Deletion of the structural PTX gene from the *B. pertussis* chromosome. To avoid possible homologous recombination between the wild-type PTX gene on the *B. pertussis* chromosome and mutated genes on incoming plasmids, the chromosomal gene was deleted from the *B. pertussis* Tohama I strain. Plasmid pTOX9 containing the complete PTX operon and its flanking regions was digested with *Kpn*I and *Bgl*II and then treated with *Bal* 31 exonuclease to remove an additional 150 to 200 bp at either end, such that the PTX promoter with its regulatory sequences (14), as well as the cistrons of subunits S1, S2, S4, and S5 and the 5′ end of the S3 cistron (Fig. 1), were removed. After digestion the DNA was treated with T4 DNA polymerase in the presence of all four nucleotides, ligated, and introduced into *E. coli* TG1 by transformation. The *E. coli* clones were then analyzed by colony blot hybridization with the two DNA probes shown in Fig. 1. Plasmid DNA of clones that hybridized with probe 2 but failed to hybridize with probe 1 was purified and analyzed by Southern blot hybridization with the same probes and by DNA sequencing with oligonucleotide S32 as a primer. The plasmid containing the *B. pertussis* DNA sequence shown in Fig. 1 was then digested with *Cla*I, and the resulting 7-kb DNA fragment containing the PTX gene-flanking regions was inserted into the unique *Hin*dIII site of pSS1129, after treatment of the DNA with T4 DNA polymerase in the presence of all four nucleotides. The recombinant plasmid, designated pRIT13295, was then used to delete the PTX operon in the *B. pertussis* chromosome.

E. coli SM10(pRIT13295) was mated with *B. pertussis*

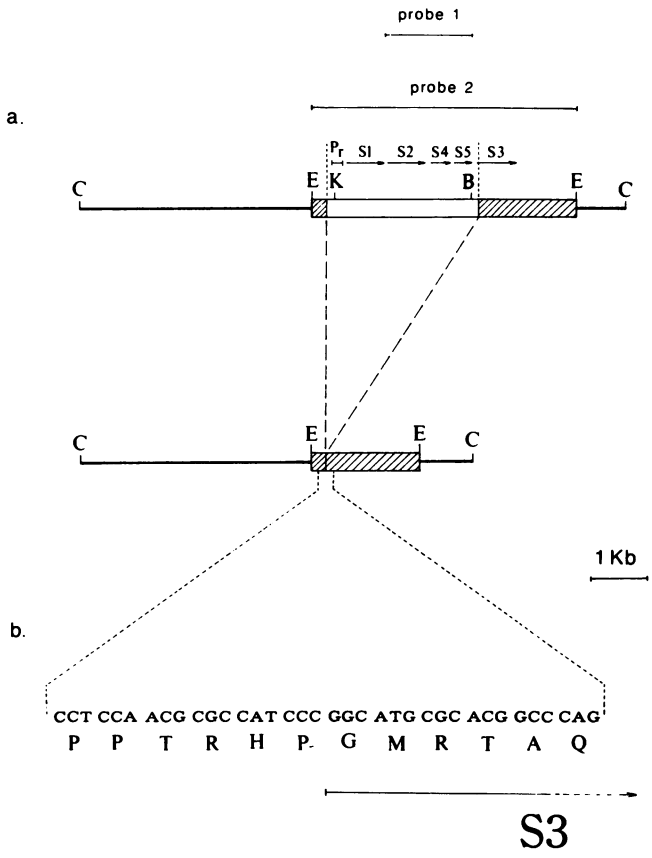

FIG. 1. Schematic representation of the PTX gene region. (a) Wild-type PTX gene and its flanking region. Probes 1 and 2 represent the lengths and positions on the PTX genes of the two probes used for hybridization. The arrows show the length, direction, and position on the chromosome of the PTX subunit cistrons. Pr indicates the promoter-regulatory region. The open box represents the portion of the PTX gene that is deleted. The hatched boxes and the solid line represent the part of the PTX gene and its flanking regions that remained after deletion. Restriction sites: C, *Cla*I; E, *Eco*RI; K, *Kpn*I; B, *Bgl*II. (b) DNA sequence and derived protein sequence of the junction after deletion. The nucleotides are grouped in triplets and in frame with the S3 subunit cistron. Amino acids are shown by the single-letter code. The remaining portion of the S3 subunit cistron is indicated by the arrow.

Tohama I (Nal^r Sm^r), a spontaneous mutant isolated by two consecutive selections of hemolytic *B. pertussis* Tohama I, first on BG agar containing nalidixic acid and then on BG agar containing streptomycin. After mating, the *B. pertussis* clones with pRIT13295 integrated into their chromosomes were selected on BG plates containing nalidixic acid and gentamicin. The gentamicin-resistant clones were then plated on BG agar containing streptomycin to select for a second homologous recombination event. After the selection steps approximately 100 hemolytic *B. pertussis* clones were analyzed by colony blot hybridization with probes 1 and 2 (Fig. 1). Chromosomal DNA from clones that hybridized with probe 2 but not with probe 1 was purified and analyzed by Southern blot hybridization after digestion with *Eco*RI or *Pst*I. Figure 2a shows a Southern blot analysis of one of these clones, designated *B. pertussis* BPRA, compared with that of *B. pertussis* Tohama I. Probe 2 (Fig. 1) hybridized to a 4.7-kb *Eco*RI DNA fragment from *B. pertussis* Tohama I, whereas it hybridized to an approximately 2-kb *Eco*RI DNA

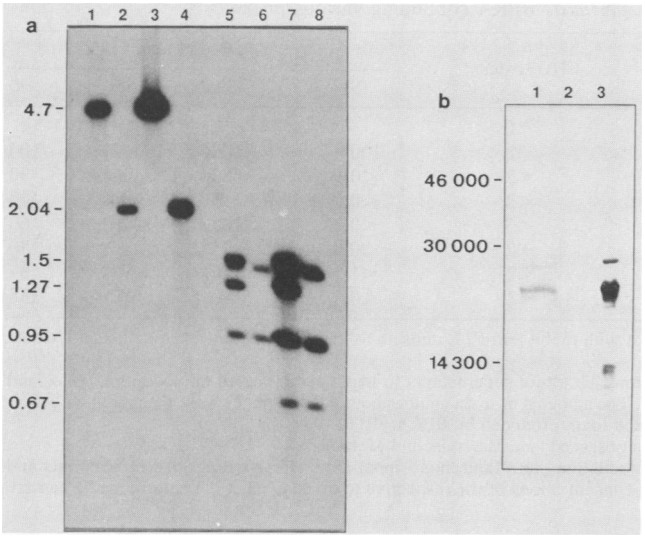

FIG. 2. Southern blot (a) and Western blot (b) analyses of *B. pertussis* Tohama I and BPRA. Southern blot analysis was performed with probe 2 (Fig. 1) hybridized with genomic DNA (1 μg per lane) from *B. pertussis* Tohama I (lanes 1 and 5) and BPRA (lanes 2 and 6), plasmid pTOX9 (20 ng per lane) (lanes 3 and 7), and pRIT13295 (20 ng per lane) (lanes 4 and 8) digested with *Eco*RI (lanes 1 through 4) or *Pst*I (lanes 5 through 8). The sizes in kilobases of some of the hybridizing DNA fragments are shown in the left margin. Western blot analysis was performed on 500 μl of trichloroacetic acid-precipitated culture supernatants of *B. pertussis* Tohama I (lane 1) and BPRA (lane 2) and 1 μg of purified PTX (lane 3) with a mixture of monoclonal anti-PTX antibodies. The antibodies used were anti-S1 5C2, anti-S2 P11B10 (12), anti-S3 F19B2, anti-S4 43H10, and anti-S5 83H1 (11). The molecular weights are shown in the left margin.

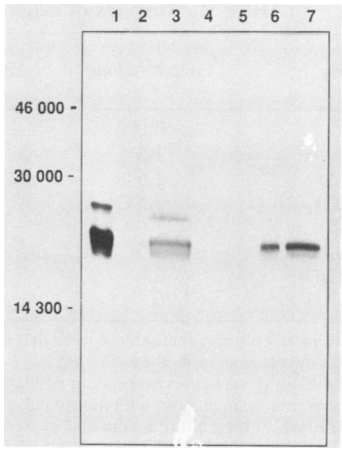

FIG. 3. Western blot analysis of PTX synthesized by *B. pertussis* BPRA containing various plasmids. After growth for 4 days in 5 ml of SS medium containing tetracycline and cyclodextrin, the *B. pertussis* BPRA cells containing pRIT13294 (lanes 2 and 3), pLAFRII (lanes 4 and 5), or pRIT13368 (lanes 6 and 7) were separated from the culture medium by centrifugation. The cells were suspended in 200 μl of PBS. Then 100 μl of triple-strength electrophoresis sample buffer (25) was added, and 10-μl samples were loaded on lanes 2, 4, and 6. The culture media were precipitated with 10% trichloroacetic acid and suspended in 100 μl of PBS. A 50-μl sample triple-strength electrophoresis sample buffer was added, and 15-μl samples were loaded on lanes 3, 5, and 7. After electrophoresis and electroblotting, the S1 and S2 subunits were detected as described previously (29) with a mixture of anti-S1 monoclonal antibody B2F8 (32) and anti-S2 monoclonal antibody P11B10 (12). Lane 1 contains 1 μg of purified PTX. Molecular weights of markers are shown in the left margin.

fragment from strain BPRA, indicating that 2.7 kb of the *Eco*RI fragment containing the PTX operon in *B. pertussis* Tohama I was deleted in strain BPRA. Southern blot analysis with the same probe on DNA digested with *Pst*I showed that the deleted fragment in BPRA originally contained the DNA coding for S1, S2, S4, and S5 subunits, as well as the promoter-regulatory region, and the 5′ end of the S3 cistron. Western blot analysis (Fig. 2b) indicated that strain BPRA did not produce any of the PTX subunits, whereas *B. pertussis* Tohama I was able to produce holotoxin under the same growth conditions. The growth rate in SS medium and the production of other virulence factors such as filamentous hemagglutinin (FHA) were not significantly altered in strain BPRA, as compared with *B. pertussis* Tohama I, indicating that at least in these growth conditions the presence of the PTX gene provides no growth advantage and does not influence FHA production.

Expression in *B. pertussis* of the PTX gene on a recombinant plasmid. Lee et al. (26) have shown that pMA2, a pLAFRII derivative containing the PTX gene, can be introduced into and will replicate in *Bordetella* spp. However, the plasmids appeared to be rather unstable in *B. pertussis* Bp357 (*ptx*$^-$::Tn*5*), which produced a small amount of cell-associated PTX, indicating that the overproduction of PTX in *B. pertussis* is detrimental to the bacterium. In our efforts to develop a *B. pertussis* expression system, it was important to investigate whether the PTX gene in pLAFRII derivatives could be stably maintained and expressed in *B. pertussis* BPRA. The 4.7-kb *Eco*RI DNA fragment containing the PTX operon (27, 36) was therefore inserted into the unique *Eco*RI site of pLAFRII, and the resulting recombinant plasmid, pRIT13294, was introduced into *B. pertussis* BPRA by conjugation. The hemolytic Tet^r *B. pertussis* clones were then grown in SS medium in the presence of tetracycline and cyclodextrin for further analysis. After growth, both the cells and the culture medium were analyzed for the presence of PTX subunits by Western blots with monoclonal anti-S1 (B2F8 [32]) and anti-S2 (P11B10 [12]) antibodies. BPRA (pRIT13294) produced and secreted PTX subunits that reacted with the monoclonal antibodies (Fig. 3). Although no anti-S3 antibody was used in this experiment, the S3 subunit was nevertheless detected because of a slight cross-reactivity of the anti-S2 antibody P11B10 with S3, most likely due to the high degree of homology between S2 and S3 (30, 36). In contrast, in BPRA(pLAFRII), no PTX subunits were detected. These results indicate that the PTX gene can be expressed from a low-copy-number plasmid in *B. pertussis* and that consequently the *vir* gene-dependent regulation acts in *trans*.

Analysis by ELISA with rabbit and mouse anti-PTX polyclonal antibodies to detect and quantify PTX indicated that BPRA(pRIT13294) secreted similar but somewhat lower amounts of PTX (3.2 μg/ml) as compared with the Tohama I strain (6.5 μg/ml). BPRA containing no plasmid or pLAFRII secreted no detectable PTX (<0.05 μg/ml).

Role of the disulfide bond in the assembly and biosynthesis of PTX. To directly investigate the role of the disulfide bond of the S1 subunit in the assembly and biosynthesis of PTX, Cys-41 of the S1 subunit was deleted or replaced by Gly or Ser. To introduce site-specific mutations in the PTX operon, the entire gene was inserted into pUC7 as an *Eco*RI DNA fragment. The resulting plasmid, pRIT13070, was then used

TABLE 2. Analyses of mutant PTX produced by *B. pertussis* BPRA containing various plasmids

BPRA containing:	Concn[a] (μg/ml)	Haptoglobin binding[b] (%)	CHO toxicity[c]	% of bound toxin[d]			
				S1	S2	S3	S4
pLAFRII	<0.008		<2				
pRIT13294	2.5	100	32	100	100	100	100
pRIT13368 (C41Δ)	1.5	88	<2	<0.9	82	117	90
pRIT13369 (C41G)	2.5	105	<2	<0.7	113	94	100
pRIT13370 (C41S)	1	74	<2	<0.9	105	120	60
pRIT13371 (S1Δ187)	1.5	74	<2	<0.9	75	76	80
pRIT13372 (S1Δ207)	2	100	<2	<1	110	93	87
pRIT13373 (S1Δ207′)	6	136	<2	<0.14	140	87	138

[a] The concentrations of culture supernatants were determined by sandwich ELISA with rabbit anti-PTX antisera as coating antibodies and mouse polyclonal anti-PTX antisera as developing antibodies.

[b] The haptoglobin-binding capacities were assayed by the addition of serial dilutions of culture supernatants to haptoglobin-coated microdilution plates and quantification of the bound toxin analogs with mouse polyclonal anti-PTX antibodies. The amounts of toxin analogs bound to the plates were compared with their respective concentrations in the culture media and expressed as percentages of bound toxin produced by BPRA(pRIT13294).

[c] Highest dilution of the culture supernatants at which CHO cell cytotoxicity was observed (see Materials and Methods).

[d] The different subunits were quantified by ELISA with haptoglobin-coated microdilution plates and monoclonal antibodies as described in Materials and Methods. The amounts of the subunits bound to the plates were compared with the concentrations of the respective toxin analogs in the culture media and are expressed as percentages of bound toxin produced by BPRA(pRIT13294).

to introduce the various mutations of the S1 subunit cistron. *E. coli* TG1 cells were infected with φRIT20003, φRIT20004, or φRIT20005, recombinant M13 phages containing DNA coding for the catalytic domain of the S1 subunit with a deletion of codon 41, its replacement by GGC coding for Gly, or AGC coding for Ser, respectively. The double-stranded replicative DNA forms were isolated and digested with *Acc*I. The 300-base-pair (bp) *Acc*I fragments were then inserted into *Acc*I-digested pRIT13070 to yield pRIT13374, pRIT13375, and pRIT13376, respectively. The modified PTX genes of these three plasmids were then isolated as 4.7-kb *Eco*RI DNA fragments and inserted into the *Eco*RI site of pLAFRII to yield pRIT13368, pRIT13369, and pRIT13370, respectively. *E. coli* SM10 was then transformed with each of the three recombinant plasmids and conjugated with *B. pertussis* BPRA. The three BPRA strains containing these plasmids were then analyzed for the production and secretion of PTX. Western blot analysis of the culture medium and the *B. pertussis* cells with anti-S1 and anti-S2 monoclonal antibodies indicated that all three mutant genes expressed PTX-cross-reactive material, but that, in contrast to the wild type gene on pRIT13294 in BPRA, no S1 subunit could be detected either in cell-associated form or in the culture supernatants. The S2 subunit was readily detected in the culture supernatant, although a significant amount remained cell associated (Fig. 3). With the ELISA, subunits S3 and S4 were also detected (Table 2). This result indicates that Cys-41 is essential for the stable synthesis of S1 in *B. pertussis*, most likely through its involvement in the unique disulfide bond, and that the S2 subunit as part of the B oligomer can be synthesized and secreted in the culture medium in the absence of the S1 subunit.

Role of the carboxy-terminal hydrophobic region in the solubility of the S1 subunit. Preliminary results indicated that a carboxy-terminal truncation of a recombinant S1 subunit produced in *E. coli* increased its solubility (3, 29). Hydropathy plots (24) of the S1 subunit indicate that the carboxy-terminal part of the molecule is rather hydrophobic. To investigate in more detail the influence of the carboxy-terminal region of the S1 subunit on its solubility, the truncated recombinant S1 subunit rS1d, which is produced in *E. coli* (29) and lacks the 47 carboxy-terminal amino acids, was compared with the full-length rS1 protein in a cell extraction analysis. *E. coli* cells producing either S1 monomer synthesized a protein that was reactive with anti-S1 monoclonal antibody B2F8 in roughly similar amounts (Fig. 4). When cells were washed with PBS or distilled water, very little rS1 leaked out from the *E. coli* cells, whereas considerable amounts of rS1d were released especially after the second, hypotonic wash. After lysis of the cells and centrifugation of the lysates, very little rS1 was detected in the supernatant fraction, and none was detected in the subsequent washes (Fig. 4). The majority of rS1 was present in the

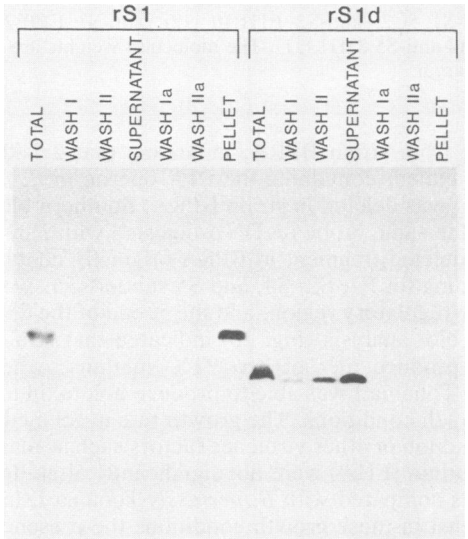

FIG. 4. Western blot analysis of the partitioning of rS1 and rS1d produced in *E. coli*. One-litre cultures of *E. coli* cells producing the indicated recombinant S1 subunit analogs were harvested by centrifugation and suspended in 10 ml of PBS (TOTAL). The cells were washed once with 10 ml of PBS (WASH I) and once with 10 ml of distiled water (WASH II). The cells were then suspended in 10 ml of 25 mM Tris hydrochloride (pH 7.6) and 25 mM NaCl and disrupted in a French pressure cell. The lysates were centrifuged, and the supernatant was analyzed. The pellets were washed twice with 10 ml of the same buffer (WASH Ia and WASH IIa), and the washed pellet was suspended in 10 ml of the same buffer (PELLET). For the Western blot analysis 0.01-ml samples of each fraction were mixed with an equal volume of double-strength electrophoresis sample buffer (25) and heated to 95°C for 5 min. After electrophoresis the proteins were transferred to nitrocellulose and probed with monoclonal anti-S1 antibody B2F8 as described elsewhere (29).

pellet fraction and could only be solubilized with 6 M urea. In contrast, the majority of rS1d was detected in the supernatant fraction of the cell lysates. Only trace amounts could be detected after the first wash, and none was detected after the second wash or in the pellet (Fig. 4). These results demonstrate the direct influence of the carboxy-terminal region on the solubility of the S1 subunit.

Closer examination of the hydropathy plot reveals the presence of two hydrophobic regions at the carboxy-terminal end of the S1 subunit, both deleted in rS1d. By using a similar cell extraction analysis, the importance of the major hydrophobic region (extending from amino acid residues 183 to 196) on solubility of S1 was analyzed. Therefore the *SalI-XbaI* DNA fragment of pTXS11 was replaced by a 300-bp *SalI-AluI* DNA fragment encoding the major hydrophobic region of S1, up to amino acid residue 207. For this construction pTXS11 (29) was first digested with *XbaI*, treated with T4 DNA polymerase in the presence of all four nucleotides, and then digested with *SalI*. pRIT13070 was digested with *SalI* and *AluI*, and the resulting 300-bp *SalI-AluI* DNA fragment was inserted into pTXS11. After ligation and transformation in *E. coli* TG1, the synthesis and solubility of this truncated S1 subunit were analyzed by Western blotting after fractionation; the truncated S1 subunit (rS1Δ207) was found in the particulate fraction (data not shown).

Role of the carboxy-terminal region in the assembly and biosynthesis of PTX. To investigate the role of the carboxy-terminal region and its hydrophobic portion on toxin biosynthesis and assembly, the S1 subunit cistron in the PTX gene was replaced by DNA coding for its rS1d analog and truncated S1 analogs containing the hydrophobic portion. pTXS11, coding for rS1d, was digested with *SalI* and *XbaI*, and the resulting 240-bp DNA fragment was inserted into *SalI-XbaI*-digested pRIT13070 to yield pRIT13377. The resulting PTX gene contained a truncated S1 cistron, coding for amino acids 1 to 187 (Fig. 5). This gene was then inserted into pLAFRII to yield pRIT13371. In addition, the 380-bp *SalI-XbaI* DNA fragment of pRIT13070 was replaced by the 300-bp *SalI-AluI* fragment described above. After ligation, transformation, and DNA sequencing, one plasmid, named pRIT13378, was found to contain the correct junction, with the stop codon immediately following the Ser-207 codon, whereas one plasmid, named pRIT13380, contained a reading frame shift at the junction resulting in a cistron coding for amino acid residues 1 through 207 of S1 and 11 residues from a different reading frame of the same gene (Fig. 5). Both DNA fragments were inserted into pLAFRII, yielding pRIT13372 and pRIT13373. Plasmids pRIT13371, pRIT13372, and pRIT13373 were then introduced into *B. pertussis* BPRA, and the expression products were analyzed by Western blotting with anti-S1 and anti-S2 monoclonal antibodies. As in the case of the mutant proteins containing alterations in the Cys-41 position of S1, the carboxy-terminal deletions resulted in no detectable S1 subunit in the culture supernatant or in a cell-associated form, whereas S2 was efficiently synthesized and secreted. These results indicate that, in addition to the disulfide bond, the carboxy-terminal region of the S1 subunit is essential for the stable expression of the S1 cistron and the secretion of S1 in the culture medium. Furthermore, hydrophobicity alone is not responsible for the interaction of S1 with the B oligomer, and probably more precise interactions involving residues downstream of amino acid 207 are necessary.

Analysis of the mutant PTX analogs. By using an ELISA, the mutant proteins were analyzed for their ability to bind to

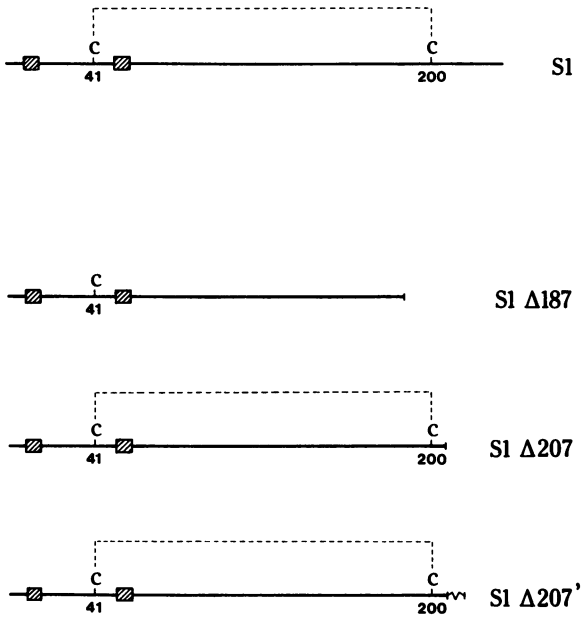

FIG. 5. Schematic representation of the carboxy-terminally truncated S1 subunits. The solid line S1 represents the length of the entire S1 subunit. The hatched boxes show two regions of the S1 subunit with sequence homology to the cholera toxin A subunit. C's above the numbers 41 and 200 show the positions of the two cysteine residues of S1. The interrupted line indicates the disulfide bond linking Cys-41 to Cys-200. S1Δ187 represents the length of the S1 molecule from which amino acid residues 187 to 234 were deleted and therefore lacking Cys-200 and the disulfide bond. S1Δ207 and S1Δ207′ represent the S1 molecules from which amino acid residues 207 to 234 were deleted. S1Δ207′ contains in addition 11 amino acids from a different reading frame, indicated by the wavy line. The following are the carboxy-terminal sequences starting at position 180 (for S1Δ187) or 200 (for S1Δ207 and S1Δ207′) with the uppercase letters indicating the amino acid residues of S1: . . . .SRRSVASII (S1Δ187), . . .CMARQAES (S1Δ207), and . . .CMARQAESrpgpap pnsgn (S1Δ207′).

haptoglobin, as a model system for the interaction of PTX with its receptors (11). Serial dilutions of the culture supernatants from BPRA containing no plasmid, pLARFRII, or pLAFRII derivatives with the wild-type PTX gene or the mutant genes were incubated on haptoglobin-coated microdilution plates, and the amounts of haptoglobin-bound material were detected with polyclonal anti-PTX antibodies. The absorbance in the linear range was then compared with the concentration of the various PTX proteins and expressed in percent binding as compared with that of the wild-type molecule. All mutant PTX proteins showed similar haptoglobin-binding capacity as compared with the wild type [BPRA(pRIT13294)] (Table 2), suggesting the functional integrity of the B oligomer and indicating that the absence of the S1 subunit does not appear to modify the ability of PTX to bind to haptoglobin.

The cytotoxicity of the proteins was measured by using the CHO cell assay. None of the mutant proteins showed any significant CHO cell cytotoxicity (Table 2), whereas the culture supernatant fraction of BPRA(pRIT13294) was cytotoxic up to a 32-fold dilution. This result indicates that, since the receptor-binding site of the mutant proteins is probably not affected, the absence of the S1 subunit causes the loss of the CHO cell cytotoxicity, which confirms the findings by Burns et al. (7) that CHO cell cytotoxicity is dependent on ADP ribosylation by the S1 subunit.

To further study the integrity of the secreted B oligomers and the absence of the S1 subunit, ELISAs were performed on serial dilutions of the *B. pertussis* culture supernatants. After binding to haptoglobin-coated microdilution plates, the PTX proteins were detected and quantified by using monoclonal antibodies specific for S1, S2, S3, and S4. None of the mutant proteins contained any detectable S1 subunit. On the other hand, all proteins contained stoechiometrically comparable S2, S3, and S4 subunits and were in that respect very similar to the PTX protein synthesized and secreted by BPRA(pRIT13294), indicating that the absence of S1 did not alter the composition of the B oligomer and strongly suggesting that the B oligomer was secreted in an assembled form in the absence of S1. ELISAs with anti-S5 monoclonal antibodies (11) did not allow the reproducible quantification of the S5 subunit in the mutant proteins or the parents toxin, presumably because S5 may be buried in the holotoxin molecule and not be readily accessible to antibodies (40).

DISCUSSION

PTX, the most complex bacterial protein exotoxin studied so far, has attracted much attention lately because of its potential as an essential component in the new acellular pertussis vaccines (1). Much effort has been focused on the elucidation of the enzymatic mechanism and the identification of essential residues in the ADP ribosyltransferase activity of the S1 subunit (3, 5, 28, 37). In contrast, little is known about the assembly of the different subunits, the regions important for these interactions, and the biosynthesis of the holotoxin. Although cloning vectors have been developed (39) that are useful for the study of the holotoxin biosynthesis by recombinant DNA technology, these systems are time consuming and labor intensive when a large number of mutants are analyzed. Therefore a simplified system was developed based on the expression of PTX and PTX-cross-reactive mutant genes on low-copy-number, broad-host-range plasmids. With plasmid pLAFRII-derived vectors used in this study, the PTX gene was expressed at levels comparable to those of the chromosomal gene in *B. pertussis* Tohama I. Although pLAFRII-derived plasmids could be present in more than one copy per cell (10), overexpression of the PTX gene was not achieved, consistent with the idea that overproduction of PTX may be detrimental to *B. pertussis* (26). Since even enzymatically inactive mutant proteins (after deletion of Cys-41) were not overproduced, this deleterious effect is independent of the ADP ribosyltransferase activity of S1. It may be due to the activities of the B oligomer, possibly through such mechanisms as channel formation in the *B. pertussis* membranes. Alternatively, the lack of overproduction may be the result of a gene dosage effect in the mechanism of the *vir*-dependent PTX gene expression. Studies are in progress to investigate whether the functional PTX gene on pLAFRII derivatives has an effect on the plasmid copy number.

By using the developed cloning system, the effect of the disulfide bond in S1 on the biosynthesis and the in vivo assembly of the holotoxin molecule was examined. When Cys-41 of S1 was deleted by site-directed mutagenesis, no S1 subunit was detected in the culture supernatant by Western blot analysis or ELISA with anti-S1 monoclonal antibodies. It can be ruled out that the absence of reaction in Western blots and ELISAs was due to the destruction of the epitopes on S1 by the mutation, since the same mutation in recombinant S1 produced in *E. coli* did not alter the reactivity of the S1 analog with these monoclonal antibodies (Locht et al., in press). The same effect was observed when Cys-41 was replaced by the isosteric serine residue or by glycine. Glycine was chosen because this substitution resulted in consistently higher residual enzymatic S1-specific activity than any other substitution, including serine. This, together with the fact that all these mutant proteins, when synthesized in *E. coli*, react with a panel of monoclonal antibodies that are specific for conformational epitopes (11), suggests that no important conformational changes had occurred by the amino acid substitutions. Truncation of the carboxy-terminal region of S1 up to residue 187 or to residue 207, including the second cysteine (Cys-200), which is essential for the formation of the intrachain disulfide bond, had the same effect as modification of Cys-41. The S1 subunit was not detectable with monoclonal antibodies in any of the mutants tested, although the same truncations of recombinant S1 analogs synthesized in *E. coli* yielded molecules that were reactive with all (more than 50) anti-S1 monoclonal antibodies tested so far (11). Furthermore, recombinant truncated S1 produced in *E. coli* expressed enzymatic NAD glycohydrolase activity indistinguishable from that of the full-length molecule, indicating that the truncation of the carboxy-terminal region does not alter the overall tertiary structure in an important way (3, 29).

The lack of detectable S1 in the culture supernatant in the absence of its disulfide bond or when its carboxy-terminal region is deleted indicates a defect in the binding of S1 subunit with the B oligomer. Burns and Manclark (9) have recently shown that after reduction of the disulfide bond of the isolated S1 subunit its in vitro reassociation with the B oligomer was affected. This binding to the B oligomer appears thus to be important for the secretion of the S1 subunit in the culture medium, suggesting that individual subunits are not secreted from *B. pertussis*. This is consistent with the results of Marchitto et al. (32), who showed that insertion of a Tn*5* transposon (43) in the S3 subunit cistron (30) resulted in the lack of secretion of the PTX subunits into the culture medium, although some subunits could be detected when solubilized whole cellular material was analyzed by Western blotting.

Whereas S1 was barely detectable, the S2 subunit appeared to accumulate probably in the periplasmic space of the Tn*5* mutants. We therefore thought that the holotoxin had to be assembled in the periplasmic space before it was secreted into the culture medium. However, functional B oligomer could be assembled and secreted in the absence of S1 (Fig. 3). The fact that significant amounts of S2 in the mutant molecules are still cell associated as compared with the wild-type holotoxin may suggest that secretion of the B oligomer is not as efficient in the absence of S1 as in its presence. Surprisingly, no S1 could be detected in cell-associated form. It has been reported that the biogenesis of S2 and S4 requires the uninterrupted expression of the S1 cistron (4). It is unlikely that the absence of S1 in the mutant proteins is due to the lack of the transcription or translation caused by the mutations, since the other cistrons of the operon are expressed. Furthermore, in other bacterial expression systems no decrease in the S1 gene expression level was observed when the Cys-41 codon was mutated (Fig. 4). On the other hand, because of the absence of the disulfide bond or the deletion of the carboxy-terminal region and the resulting deficiency in assembly of S1 with the B oligomer, the unassociated S1 may readily be degraded after or during translation and translocation through the inner membrane of *B. pertussis*. It is known that the isolated S1 subunit is more rapidly digested by proteases than S1, which

is associated with the B oligomer (6). This situation may be very similar to that of the *E. coli* heat-labile toxin, in which the enzymatically active A subunit is rapidly degraded in the periplasmic space of *E. coli* during biosynthesis (16). Future work with pulse-chase experiments may help to clarify the question concerning the turnover rate of the S1 subunit in the absence of the disulfide bond and the carboxy-terminal region.

Black and Falkow (4) reported that mutations in the S5 subunit cistron adversely affected the synthesis or stability of S1. The reasons for this instability were not clear. It is likely that in the absence of S5 the S1 subunit cannot be assembled into a holotoxin molecule; consistent with our findings, the unbound S1 subunit is then rapidly degraded in the periplasmic space. It may be possible that the association of the S1 subunit with the B oligomer occurs via its direct interaction with S5. Alternatively, the S5 subunit may act as a connecting subunit between the two dimers (S2-S4 and S3-S4); in the absence of S5, no assembled B oligomer is formed (40) and no association of S1 with the B oligomer would be possible.

Under conditions of low ionic strength and neutral pH, the majority of a full-length recombinant S1 subunit remained associated with the particulate fraction, whereas a truncated recombinant S1 subunit (rS1d) appeared in the soluble fraction of the disrupted cells. The differential partitioning cannot be attributed to differences in the amounts of protein produced by the two expression plasmids and likely reflects the relatively hydrophobic character of the 47 carboxy-terminal amino acids and their influence on solubility via the promotion of self-aggregation or association with other molecules. Inclusion of the hydrophobic amino acids 188 to 207 in S1Δ207 and S1Δ207′ rendered the truncated S1 subunits insoluble under the same conditions. Although the insolubility of the S1 molecule could therefore be attributed to the major hydrophobic region including amino acid residues 188 to 207, this hydrophobicity was not sufficient for the assembly of S1 with the B oligomer and its secretion in the culture medium. Although it cannot be ruled out that the hydrophobic region plays a role in binding of the S1 subunit to the B oligomer, other residues downstream of amino acid 207 are essential for this interaction. Experiments are now under way to more precisely define these residues.

The B-oligomer molecules isolated from the described culture media contain no detectable S1, as evidenced by ELISA and CHO cell assays. Antibodies raised in mice against purified B oligomer are able to neutralize PTX-mediated cytotoxicity and to provide protection against challenge with PTX. Therefore the B oligomer was proposed as a possible component candidate for a safer new vaccine against whooping cough (2). However S1-free B oligomer is difficult to prepare, and many biochemically purified B-oligomer preparations contain detectable amounts of S1 (data not shown). In addition, the conditions described for the purification of B oligomer are partially denaturing because of the presence of urea in the buffers used (40). The genetic alteration of Cys-41 or the carboxy-terminal region of the S1 subunit cistron as described in this paper may therefore provide a useful alternative for the production and purification of the B oligomer of PTX.

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LITERATURE CITED

1. **Ad hoc group for the study of pertussis vaccines.** 1988. Placebo-controlled trial of two acellular pertussis vaccines in Sweden—protective efficacy and adverse events. Lancet **ii**:955–960.
2. **Arciniega, J. L., D. L. Burns, E. Garcia-Ortigoza, and C. R. Manclark.** 1987. Immune response to the B oligomer of pertussis toxin. Infect. Immun. **55**:1132–1136.
3. **Barbieri, J. T., and G. Cortina.** 1988. ADP-ribosyltransferase mutations in the catalytic S-1 subunit of pertussis toxin. Infect. Immun. **56**:1934–1941.
4. **Black, W. J., and S. Falkow.** 1987. Construction and characterization of *Bordetella pertussis* toxin mutants. Infect. Immun. **55**:2465–2470.
5. **Burnette, W. N., W. Cieplak, V. L. Mar, K. T. Kaljot, H. Sato, and J. M. Keith.** 1988. Pertussis toxin S1 mutant with reduced enzyme activity and a conserved protective epitope. Science **242**:72–74.
6. **Burns, D. L., S. Z. Hausman, W. Lindner, F. A. Robey, and C. R. Manclark.** 1987. Structural characterization of pertussis toxin A subunit. J. Biol. Chem. **262**:17677–17682.
7. **Burns, D. L., J. G. Kenimer, and C. R. Manclark.** 1987. Role of the A subunit of pertussis toxin in alteration of chinese hamster ovary cell morphology. Infect. Immun. **55**:24–28.
8. **Burns, D. L., and C. R. Manclark.** 1986. Adenine nucleotides promote dissociation of pertussis toxin subunits. J. Biol. Chem. **261**:4324–4327.
9. **Burns, D. L., and C. R. Manclark.** 1989. Role of cysteine 41 of the A subunit of pertussis toxin. J. Biol. Chem. **264**:564–568.
10. **Figurski, D. H., R. F. Pohlman, D. H. Beckhofer, A. S. Prince, and C. A. Kelton.** 1982. The broad host range plasmid RK2 encodes multiple kil genes potentially lethal to *Escherichia coli* host cells. Proc. Natl. Acad. Sci. USA **79**:1935–1939.
11. **Francotte, M., C. Locht, C. Feron, C. Capiau, and M. de Wilde.** 1989. Monoclonal antibodies specific for pertussis toxin subunits and identification of the haptoglobin-binding site, p. 243–247. *In* R. A. Lerner, H. Ginsberg, R. M. Chanock, and F. Brown (ed.), Vaccines 89. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Frank, D. W., and C. D. Parker.** 1984. Interaction of monoclonal antibodies with pertussis toxin and its subunits. Infect. Immun. **46**:195–201.
13. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18**:289–296.
14. **Gross, R., and R. Rappuoli.** 1988. Positive regulation of pertussis toxin expression. Proc. Natl. Acad. Sci. USA **85**:3913–3917.
15. **Hewlett, E. L., K. T. Sauer, G. A. Myers, J. L. Cowell, and R. L. Guerrant.** 1983. Induction of a novel morphological response in Chinese hamster ovary cells by pertussis toxin. Infect. Immun. **40**:1198–1203.
16. **Hofstra, H., and B. Witholt.** 1985. Heat-labile enterotoxin in *Escherichia coli*. Kinetics of association of subunits into periplasmic holotoxin. J. Biol. Chem. **260**:16037–16044.
17. **Holmgren, J.** 1981. Actions of cholera toxin and the prevention and treatment of cholera. Nature (London) **292**:413–417.
18. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33**:933–938.
19. **Imaizumi, A., Y. Suzuki, S. Ono, H. Sato, and Y. Sato.** 1983. Effect of heptakis(2,6-*O*-dimethyl)β-cyclodextrin on the production of pertussis toxin by *Bordetella pertussis*. Infect. Immun. **41**:1138–1143.
20. **Kaslow, H. R., and D. D. Lesikar.** 1987. Sulfhydryl-alkylating reagents inactivate the NAD glycohydrolase activity of pertus-

sis toxin. Biochemistry **26**:4397–4402.

21. **Kaslow, H. R., L.-K. Lim, J. Moss, and D. D. Lesikar.** 1987. Structure-activity analysis of the activation of pertussis toxin. Biochemistry **26**:123–127.
22. **Kaslow, H. R., J. D. Schlotterbeck, V. L. Mar, and W. N. Burnette.** 1989. Alkylation of cysteine 41, but not cysteine 200, decreases the ADP-ribosyltransferase activity of the S1 subunit of pertussis toxin. J. Biol. Chem. **264**:6386–6390.
23. **Katada, T., M. Tamura, and M. Ui.** 1983. The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. Arch. Biochem. Biophys. **224**:290–298.
24. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
26. **Lee, C. K., A. Roberts, and S. Perrin.** 1989. Expression of pertussis toxin in *Bordetella bronchiseptica* and *Bordetella parapertussis* carrying recombinant plasmids. Infect. Immun. **57**:1413–1418.
27. **Locht, C., P. A. Barstad, J. E. Coligan, L. Mayer, J. J. Munoz, S. G. Smith, and J. M. Keith.** 1986. Molecular cloning of pertussis toxin genes. Nucleic Acids Res. **14**:3251–3261.
28. **Locht, C., C. Capiau, and C. Feron.** 1989. Identification of amino acid residues essential for the enzymatic activities of pertussis toxin. Proc. Natl. Acad. Sci. USA **86**:3075–3079.
29. **Locht, C., W. Cieplak, K. S. Marchitto, H. Sato, and J. M. Keith.** 1987. Activities of complete and truncated forms of pertussis toxin subunits S1 and S2 synthesized by *Escherichia coli*. Infect. Immun. **55**:2546–2553.
30. **Locht, C., and J. M. Keith.** 1986. Pertussis toxin gene: nucleotide sequence and genetic organization. Science **232**:1258–1264.
31. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Marchitto, K. S., J. J. Munoz, and J. M. Keith.** 1987. Detection of subunits of pertussis toxin in Tn5-induced *Bordetella* mutants deficient in toxin biological activity. Infect. Immun. **55**:1309–1313.
33. **Moss, J., S. J. Stanley, D. L. Burns, J. A. Hsia, D. A. Yost, G. A. Myers, and E. L. Hewlett.** 1983. Activation by thiol of the latent NAD glycohydrolase and ADP-ribosyltransferase activities of *Bordetella pertussis* toxin (islet-activating protein). J. Biol. Chem. **258**:11879–11882.
34. **Moss, J., S. J. Stanley, P. A. Watkins, D. L. Burns, C. R. Manclark, H. R. Kaslow, and E. L. Hewlett.** 1986. Stimulation of the thiol-dependent ADP-ribosyltransferase and NAD glycohydrolase activities of *Bordetella pertussis* toxin by adenine nucleotides, phospholipids, and detergents. Biochemistry **25**: 2720–2725.
35. **Munoz, J. J.** 1985. Biological Activities of Pertussigen (Pertussis Toxin), p. 1–18. *In* R. D. Sekura, J. Moss, and M. Vaughan (ed.), Pertussis toxin. Academic Press, Inc., New York.
36. **Nicosia, A., M. Perugini, C. Franzini, M. C. Casagli, M. G. Borri, G. Antoni, M. Almoni, P. Neri, G. Ratti, and R. Rappuoli.** 1986. Cloning and sequencing of the pertussis toxin genes: operon structure and gene duplication. Proc. Natl. Acad. Sci. USA **83**:4631–4635.
37. **Pizza, M., A. Bartoloni, A. Prugnola, S. Silvestri, and R. Rappuoli.** 1988. Subunit S1 of pertussis toxin: mapping of the regions essential for ADP-ribosyltransferase activity. Proc. Natl. Acad. Sci. USA **85**:7521–7525.
38. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–791.
39. **Stibitz, S., W. Black, and S. Falkow.** 1986. The construction of a cloning vector designed for gene replacement in *Bordetella pertussis*. Gene **50**:133–140.
40. **Tamura, M., K. Nogimori, S. Murai, M. Yajima, K. Ito, T. Katada, M. Ui, and S. Ishii.** 1982. Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. Biochemistry **21**:5516–5522.
41. **Tamura, M., K. Nogimori, M. Yajima, K. Ase, and M. Ui.** 1983. The role of the B-oligomer moiety of islet-activating protein, pertussis toxin, in development of the biological effects on intact cells. J. Biol. Chem. **258**:6756–6761.
42. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
43. **Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow.** 1983. Tn5-induced mutations affecting virulence factors of *Bordetella pertussis*. Infect. Immun. **42**:33–41.