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Activities of Complete and Truncated Forms of Pertussis Toxin Subunits S1 and S2 Synthesized by *Escherichia coli*

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The genes encoding the S1 and S2 subunits of pertussis toxin were expressed in *Escherichia coli* under *lac* operon transcription and translation control with pUC8 and pUC18 as the expression vectors. Various versions of the subunits were detected with anti-S1 or anti-S2 monoclonal antibodies. Recombinant S1, but not S2, subunit contained the enzymatic NAD-glycohydrolase and NAD:Gi ADP-ribosyltransferase activities. Both activities were also expressed by a truncated version of the S1 subunit in which the 48 carboxy-terminal amino acid residues, including a predicted Rossman structure and one of the two cysteines, had been deleted. The epitope for an anti-S2 monoclonal antibody was localized to the N-terminal 40-amino-acid region of the S2 subunit. Both the S1 and S2 subunits expressed in *E. coli* reacted with human hyperimmune serum. The full length and the truncated recombinant S1 subunit also reacted in Western blots with a neutralizing and protective monoclonal anti-S1 antibody. The different versions of S1 and S2 subunits expressed in *E. coli* are useful for mapping active sites, epitopes, and regions that interact with receptors or the other subunits in the holotoxin. These recombinant subunits will also facilitate the development of a safer, new-generation vaccine against whooping cough.

Harmful side effects experienced by children after inoculation with the current pertussis vaccines have contributed to diminishing parental confidence in vaccines and therefore undermine successful childhood vaccination programs. This fact has prompted several laboratories to investigate ways of developing a safer, new-generation vaccine to protect against whooping cough. One very promising strategy involves the use of recombinant DNA technology to produce nontoxic protective antigens. We chose this approach for the production of pertussis toxin (PTX; also called lymphocytosis-promoting factor, histamine-sensitizing factor, islet-activating protein, and pertussigen), a likely candidate for a new vaccine. This toxin is thought to play a major role in the pathogenesis of whooping cough (for a recent review, see reference 33). Accordingly, the toxin is also believed to be the major protective antigen and is included in new acellular pertussis vaccines (28). In mice, vaccines prepared from avirulent or toxin-deficient strains are much less protective than those prepared from virulent strains (31). Moreover, purified PTX alone offers very good protection (20) against challenge with *Bordetella pertussis*, the etiologic agent of whooping cough.

PTX is a hexameric protein composed of five dissimilar subunits, termed S1 through S5 according to their decreasing molecular weights (26,024 for S1; 21,924 for S2; 21,873 for S3; 12,058 for S4; and 11,759 for S5) (2, 14, 23, 29), in a molar ratio of 1:1:1:2:1 for the S1-S2-S3-S4-S5 oligomer (29). S1 displays an ADP-ribosyltransferase activity (11). S2 through S5 are target cell receptor-binding subunits (30) that allow S1 to enter the cells and ADP-ribosylate GTP-binding proteins responsible for signal transduction. These target proteins (transducin, Gi, and Go) vary in function and cellular distribution; thus, the toxin exerts many different pathophysiologic effects (8, 22, 32). The complete toxin gene has recently been cloned (13, 23) and sequenced (14, 23). However, our initial attempts to express this gene in *Escherichia coli* were unsuccessful. We attributed this lack of expression to one or more of the following potential difficulties: (i) the promoter of the PTX gene is inefficient in *E. coli*; (ii) putative ribosome-binding sites upstream of the initiation codons of the subunits are not optimal for translation in *E. coli*; (iii) some of the signal peptides of the PTX subunits contain cysteines which may interfere with proper transport through the inner membrane and therefore cause translation arrest; (iv) the codon usage does not resemble the codon usage of highly expressed genes in *E. coli* (14). To circumvent the first three problems, we chose to express the mature forms of these proteins under the inducible *lac* promoter and the β-galactosidase ribosome-binding sites in the pUC vectors (34).

We concentrated our initial efforts on the S1 and S2 subunits for several reasons. First, since S1 is the enzymatically active subunit, some anti-S1 antibodies may have neutralizing activities in mouse challenge experiments. A neutralizing anti-S1 monoclonal antibody has indeed been described (26). Second, dimeric complexes of S2 and S4 (dimer I) but not S3 and S4 (dimer II) (29) bind to haptoglobin (2). Since haptoglobin, like many other sialoglycoproteins, is believed to mimic target cell receptors (8), it is likely that S2 is involved in target cell receptor binding. Finally, of the five subunits in PTX, S1 and S2 yield the most intense reaction with either rabbit or mouse anti-PTX antibodies in Western blot (immunoblot) analyses (16).

MATERIALS AND METHODS

Materials. The isolation of plasmid pPTX42 has been described previously (13). Monoclonal anti-S2 antibody P11B10 (4) was kindly provided by C. D. Parker (University of Missouri, Columbia). Monoclonal anti-S1 antibodies B2F8 and 1B7 have been described elsewhere (16, 26). PTX

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was purified as described previously (21). Chinese hamster ovary (CHO-K1) cells (ATCC CCL 61) were obtained from the American Type Culture Collection (Rockville, Md.). Freund complete adjuvant, tissue culture medium (Ham's F-12), antibiotics, and L-glutamine were from GIBCO Laboratories (Grand Island, N.Y.). [*carbonyl*-^{14}C]NAD (44 mCi/mmol) was obtained from Amersham Corp. (Arlington Heights, Ill.). [*adenylate*-^{32}P]NAD (17.57 Ci/mmol) and ^{125}I-labeled rabbit anti-mouse immunoglobulin G were from New England Nuclear Corp. (Boston, Mass.). Human hyperimmune serum (Hypertussis, a solution of gamma globulins from humans hyperimmunized with phase I pertussis vaccine) was obtained from Cutter Biological Laboratories (Berkeley, Calif.). Goat anti-human and peroxidase-linked rabbit anti-goat immunoglobulin G were purchased from Cooper Biomedical, Inc. (Malvern, Pa.). *E. coli* extract for background reduction (ProtoBlot) was obtained from Promega Biotec (Madison, Wis.). Low-melting-point agarose, *E. coli* JM101 and JM109, plasmids pUC18 and pUC8, isopropyl-β-D-thiogalactopyranoside (IPTG), and restriction enzymes were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Elutip-d columns were purchased from Schleicher & Schuell, Inc. (Keene, N.H.) and used as recommended by the supplier. Nitrocellulose membranes and ion-exchange resin AG1-X2 were purchased from Bio-Rad Laboratories (Richmond, Calif.). Ovalbumin (grade V), Tween 20, concentrated Antifoam A, 3-[(3-cholamidopropyl)-dimethylammonio]1-propane-sulfonate (CHAPS), and 4-chloro-1-naphthol were purchased from Sigma Chemical Co. (St. Louis, Mo.). *B. pertussis* 18323 was kindly provided by J. L. Cowell (Food and Drug Administration, Bethesda, Md.).

Expression of PTX subunit genes in *E. coli*. The recombinant DNA techniques used have been described previously (15), except for *E. coli* transformations, which were performed as described elsewhere (5). *E. coli* cells carrying recombinant plasmids were grown at 37°C in LB medium (15) containing 250 μg of carbenicillin per ml until the optical density at 650 nm of the culture reached 0.5 IPTG was then added to a final concentration of 1 mM, and growth was continued overnight at 37°C with constant shaking. Cells were harvested by centrifugation and suspended in 0.1 volume of phosphate-buffered saline (PBS). A 0.5 volume of 3× Laemmli buffer (0.1875 M Tris hydrochloride [pH 6.8], 6% sodium dodecyl sulfate [SDS], 30% glycerol, 15% β-mercaptoethanol) (12) was added, and the samples were heated at 95°C for 5 min before loading on an SDS-12% polyacrylamide gel, with a 3% stacking gel. Electrophoresis was done under constant current (25 mA per gel for 3 h). Proteins were then transferred onto nitrocellulose filters by electroblotting (Western blotting) (1) at 0.95 A for 2 h in 25 mM sodium phosphate buffer (pH 7.4). Filters were then washed in PBS containing 5% (wt/vol) nonfat dry milk, 0.01% (vol/vol) Antifoam A, and 0.0001% (wt/vol) merthiolate (BLOTTO) (10) for 1 h at room temperature and incubated with monoclonal antibodies (1/10 dilution of culture supernatants) in BLOTTO overnight at 4°C. After the mixture was washed three times for 10 min each with BLOTTO, ^{125}I-labeled rabbit anti-mouse secondary antibodies at a 1/1,000 dilution were added in BLOTTO and incubation continued for 2 h at room temperature. The filters were washed six times for 15 min each with BLOTTO, dried, and exposed to Kodak X-Omat X-ray film overnight at −70°C.

Human hyperimmune antiserum was used to detect PTX subunits on Western blots. After electroblotting, filters were incubated at 4°C overnight in PBS containing 0.01% Tween 20 (PBS-Tween), with 0.01 volume of human hyperimmune serum and 0.02 volume of *E. coli* cell lysate (ProtoBlot). Filters were then washed three times in PBS-Tween and incubated at room temperature for 3 h in PBS-Tween containing 0.002 volume of goat antihuman antibodies. After three washes in PBS-Tween, filters were incubated for 3 h in PBS-Tween containing 0.001 volume of peroxidase-linked rabbit antigoat antibodies and then washed six times with PBS-Tween. The filters were developed in 30 ml of PBS containing 15 mg of 4-chloro-1-naphthol (dissolved in 5 ml of methanol) and 10 μl of hydrogen peroxide and finally rinsed in distilled water and dried at room temperature.

Preparation of recombinant PTX subunits. PTX subunits were expressed in 1-liter cultures as described above. After overnight induction with IPTG, cells were harvested by centrifugation at 4,000 × *g* for 20 min and washed once with 15 ml of PBS and once with 10 ml of water containing 1 mM dithiothreitol (DTT). Cells were then resuspended in 10 ml of water containing 1 mM DTT and disrupted by sequential passage through a French pressure cell, once at 4,000 lb/in^2 and twice at 10,000 lb/in^2. Cell homogenates (from *E. coli* containing pTXS11, pTXS13, or pTXS24) were fractionated by centrifugation at 12,000 × *g* for 20 min. The pellets were washed twice with 10 ml of 25 mM Tris hydrochloride (pH 7.4)–25 mM NaCl–0.1% Triton X-100–1 mM DTT. The initial supernatant fractions were dialyzed against washing buffer without Triton X-100. The pellets were then resuspended in the same buffer containing 8 M urea and 5% β-mercaptoethanol instead of DTT, incubated overnight at 37°C, and then centrifuged for 30 min at 15,000 × *g*. The supernatants were dialyzed overnight against the washing buffer containing no Triton X-100 and then centrifuged again at 15,000 × *g* for 30 min before use.

Preparation of CHO-K1 membranes. Chinese hamster ovary (CHO-K1) cells were maintained in monolayer culture by serial passage in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM L-glutamine. For the preparation of crude membranes, the cells were detached from growth vessels (dishes or roller bottles) with a rubber policeman, suspended in Dulbecco PBS (pH 7.2), and then sedimented by centrifugation at 1,000 × *g* for 10 min. The cells were suspended in 20 volumes of ice-cold 25 mM Tris hydrochloride (pH 7.5) containing 5 mM $MgCl_2$ and allowed to equilibrate for 15 min on ice. The cells were then homogenized with 30 strokes in a Dounce homogenizer equipped with a tight-fitting pestle. The homogenate was centrifuged at 600 × *g* for 10 min at 4°C to remove nuclei and intact cells. The nuclear material was extracted once more in a small volume of equilibration buffer with a few additional strokes, and the residual nuclei and unbroken cells were removed by centrifugation. The combined postnuclear supernatant fractions were centrifuged at 18,000 × *g* for 7 min to yield a microsomal or membrane pellet. The pelleted material was suspended in 50 mM Tris hydrochloride (pH 8.0) and centrifuged at 18,000 × *g* for 7 min. The final washed membrane pellet was suspended in 50 mM Tris hydrochloride (pH 8.0) at a concentration of 1 mg of protein per ml and stored at −70°C until used.

NAD-glycohydrolase and ADP-ribosyltransferase assays. Hydrolysis of NAD was measured as the release of ^{14}C-labeled nicotinamide from [*carbonyl*-^{14}C]NAD (3, 19). Reaction mixtures (100 μl) containing 40 μM [*carbonyl*-^{14}C]NAD (0.06 μCi), 20 mM DTT, 1.0% CHAPS, 100 μM ATP, 1 mg of ovalbumin per ml, 50 mM Tris hydrochloride (pH 8.0), and recombinant material (25 μl) were incubated at 30°C for

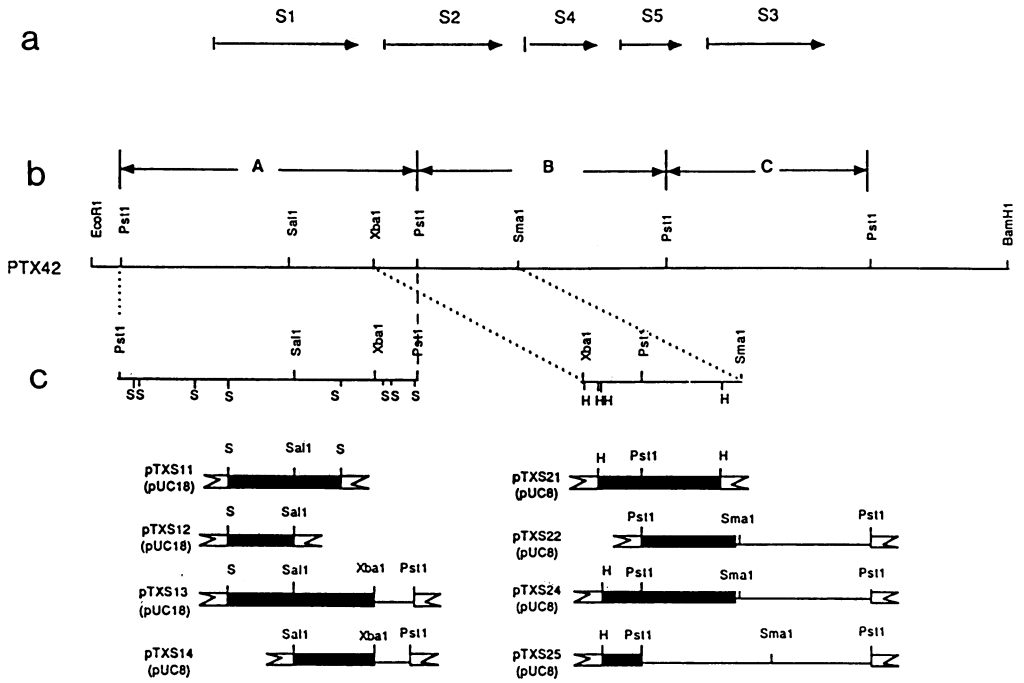

FIG. 1. Construction of PTX S1 and S2 subunit expression vectors. (a) Protein-coding regions of the mature PTX subunits. The arrows represent the length and translational directions of the subunit cistrons. (b) Partial restriction map of the PTX gene inserted in pPTX42 (13). (c) Partial restriction map of the DNA fragments used for the construction of S1 and S2 subunit expression vectors. White interrupted bars represent pUC vector DNA, black bars represent subunit coding regions, and thin lines represent *B. pertussis* DNA not coding for PTX subunits. The pUC vectors used to construct the different expression plasmids are indicated in parentheses. The expression vectors have been constructed as described in the text. S, *Sau*3A; H, *Hae*III.

8 h. Duplicate 50-μl samples were immediately mixed with 100 μl of 25 mM potassium tetraborate containing 30% (vol/vol) AG1-X2 anion-exchange resin and 15% (vol/vol) ethanol. The ion-exchange resin was sedimented by centrifugation at 15,000 × *g* for 2 min, and the radioactivity in 80 μl of the supernatant fractions was determined by liquid scintillation spectrometry.

ADP-ribosyltransferase activity was determined as the ability to catalyze the transfer of labeled ADP-ribose from [*adenylate*-^{32}P]NAD to the M_r-41,000 Gi protein in CHO-K1 membranes. Reaction mixtures (100 μl) containing 32 μM [*adenylate*-^{32}P]NAD (2 μCi), 10 mM thymidine, 100 μM ATP, 20 mM DTT, 100 μM GTP, 50 μg of CHO-K1 membrane proteins, 50 mM Tris hydrochloride (pH 8.0), and PTX or recombinant material as the enzyme source were incubated at 37°C for 30 min. The reactions were terminated by the addition of 1 ml of ice-cold 50 mM Tris hydrochloride (pH 8.0), and the membranes were sedimented by centrifugation at 15,000 × *g* for 8 min at 4°C. The membrane pellet was resuspended in ice-cold Tris hydrochloride (pH 8.0) and sedimented once more by centrifugation before solubilization in 50 μl of electrophoresis sample buffer (12) containing 5% 2-mercaptoethanol. The samples were heated to 95°C for 5 min and then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography, employing Kodak X-Omat X-ray film and Cronex Lightning-Plus intensifying screens.

Determination of anti-recombinant S2 immune response in mice. Two groups of 10 female CFW mice (3 weeks old) were immunized with either 150 or 300 μg of the recombinant S2 preparation (pTXS24 extract) in Freund complete adjuvant. The mice were boosted twice with the same amount of antigen 14 and 30 days after immunization. Three mice of each group were bled 4 days after the last booster. All mice were challenged intracranially with 30,000 to 40,000 CFU of *B. pertussis* 18323 7 days after the last booster dose. The sera from three mice in each group were assayed by radioimmunoassay with PTX-coated 96-well plates (16) and ^{125}I-labeled rabbit anti-mouse immunoglobulin G as the secondary antibody and for inhibition of PTX-mediated Chinese hamster ovary (CHO) cell clustering (6).

RESULTS

Construction of PTX subunit expression vectors. The DNA sequence and the deduced protein sequences of the PTX gene (14) were used to construct plasmids for the expression of the genes encoding PTX subunits S1 and S2 as proteins with minimal fusions to the amino-terminal residues of β-galactosidase. We used pUC18 for the expression of the S1 gene in pTXS11, pTXS12, and pTXS13 and pUC8 for the expression of S1 in pTXS14 and all versions of S2. A construction map of these vectors is shown in Fig. 1.

*Pst*I fragment A (Fig. 1) was isolated from pPTX42 by Elutip-d chromatography after complete *Pst*I digestion of the plasmid and low-melting-point agarose gel electrophoresis. This fragment was digested with *Sau*3A and ligated into pUC18 previously digested with *Bam*HI. The resulting plasmid (pTXS11) was then digested with *Sal*I and religated to construct pTXS12. *Pst*I fragment A was digested with *Sal*I, and the *Sal*I-*Pst*I fragments were ligated into pTXS12 previously digested with *Sal*I and *Pst*I. This resulted in the creation of pTXS13. The expressed S1 polypeptides from these three plasmids all contained six amino-terminal amino acids from β-galactosidase followed by five amino acids encoded by the polylinker. To construct pTXS14, we ligated

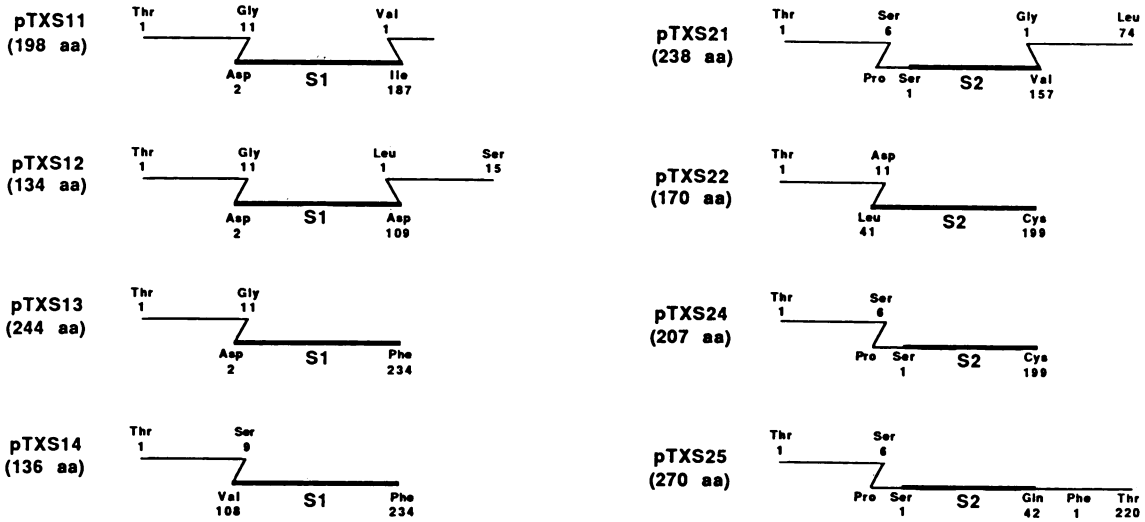

FIG. 2. Summary of the various constructs of S1 and S2. Bold lines represent amino acids (aa) encoded by the S1 and S2 subunit structural genes. Thinner lines represent amino acid sequences encoded by pUC8 or pUC19. The total length of each construct is indicated in parentheses.

the *Sal*I-*Pst*I fragment into pUC8 previously digested with *Sal*I and *Pst*I. The S1 peptide synthesized from this plasmid contained six amino acids from β-galactosidase and four amino acids encoded by the polylinker.

To construct the S2 expression vectors, the unique *Xba*I-*Sma*I fragment was isolated from pPTX42 and digested with *Hae*III. The longest *Hae*III fragment was purified and inserted into the *Sma*I site of pUC8. The resulting pTXS21 was then digested with *Pst*I and ligated to the isolated *Pst*I fragment B from pPTX42. This yielded pTXS24, with *Pst*I fragment B in the forward orientation, and pTXS25, with *Pst*I fragment B in the reverse orientation relative to *Pst*I fragment A. For the construction of pTXS22, *Pst*I fragment B was inserted into the *Pst*I site of pUC8. The S2 peptides derived from these vectors all contain six amino acids from β-galactosidase. pTXS22 also contains six amino acids encoded by the polylinker. All constructions were verified by DNA sequencing by the dideoxy chain termination method (25). The polypeptides encoded by the various S1 and S2 constructs are schematically depicted in Fig. 2.

Synthesis of S1 and S2 subunits by *E. coli*. After induction with IPTG, whole lysates of *E. coli* cells harboring either S1 or S2 subunit expression vectors were analyzed by SDS-PAGE and Coomassie blue staining (Fig. 3A). Analysis of the S1 subunit expression clones failed to reveal recombinant proteins as judged by protein staining in gels, except for a faint band detectable in clone pTXS11 (Fig. 3A). In contrast, analysis of the S2 subunit expression clones revealed that all four clones synthesized a unique polypeptide in amounts sufficient to be detected by Coomassie blue staining. The calculated M_rs of these polypeptides were consistent with the predicted sizes of their respective fusion constructs (Fig. 3A). Western blot analyses with monoclonal antibodies against S1 (B2F8) and S2 (P11B10) subunits showed that the induced proteins reacted with the appropriate monoclonal antibody (Fig. 3B). The immunoreactive product of pTXS11 was found almost exclusively in the initial supernatant fraction, whereas those of the remaining clones were detected in the pellet fraction. Expression of pTXS13 was somewhat increased when *E. coli* JM109 was used instead of JM101 (data not shown). These results indicate that the genes for individual subunits of PTX can be expressed in *E. coli* despite the putatively unfavorable codon usage.

Clones containing pTXS11 and pTXS13 both synthesized a polypeptide that reacted with monoclonal antibody B2F8, indicating that the S1 epitope for this antibody is located within the first 187 amino acids of the protein. In the cells containing pTXS13, a second, smaller polypeptide also reacted with the antibody. This polypeptide migrates at the same position as a partially degraded form of S1 derived from *B. pertussis* (data not shown). Clones containing either pTXS12 or pTXS14 did not express proteins that reacted with the antibody. However, the polypeptides encoded by these plasmids may not be sufficiently stable to be detected, since smaller recombinant polypeptides tend to be easily degraded in heterologous hosts (17).

The polypeptides encoded by pTXS11 and pTXS13 also reacted in Western blots with monoclonal anti-S1 antibody 1B7 (Fig. 4). This antibody has been shown to neutralize the biological activities of PTX (27). In addition, this monoclonal antibody is capable of conferring protection in mice to challenge with *B. pertussis*. Therefore, both of the polypeptides encoded by pTXS11 and pTXS13 retain an epitope that elicits a protective antibody response in mice.

All of the recombinant S2 polypeptides, on the other hand, appear to be sufficiently stable to be detected by both Coomassie blue staining and Western blotting (Fig. 3 and 5). Only pTXS21, pTXS24, and pTXS25 express polypeptides that react with monoclonal antibody P11B10 (Fig. 3B), which indicates that this monoclonal antibody recognizes an epitope that is located within the first 40 amino acids of the S2 subunit.

Enzymatic activities of S1 subunits synthesized by *E. coli*. Purified PTX and extracts of *E. coli* cells containing pTXS11, pTXS13, or pTXS24 were assayed for specific ADP-ribosyltransferase activity. Polypeptides encoded by pTXS11 and pTXS13 were able to specifically ADP-ribosylate the M_r-41,000 protein in the CHO-K1 cell membranes, which corresponds to the α-subunit of the GTP-binding Gi protein (Fig. 5) (8). No ADP-ribosylation of this protein could be detected in reaction mixtures lacking *E. coli* extracts or in reaction mixtures containing *E. coli*-expressed S2 subunit (pTXS24). Extracts from IPTG-induced *E. coli*

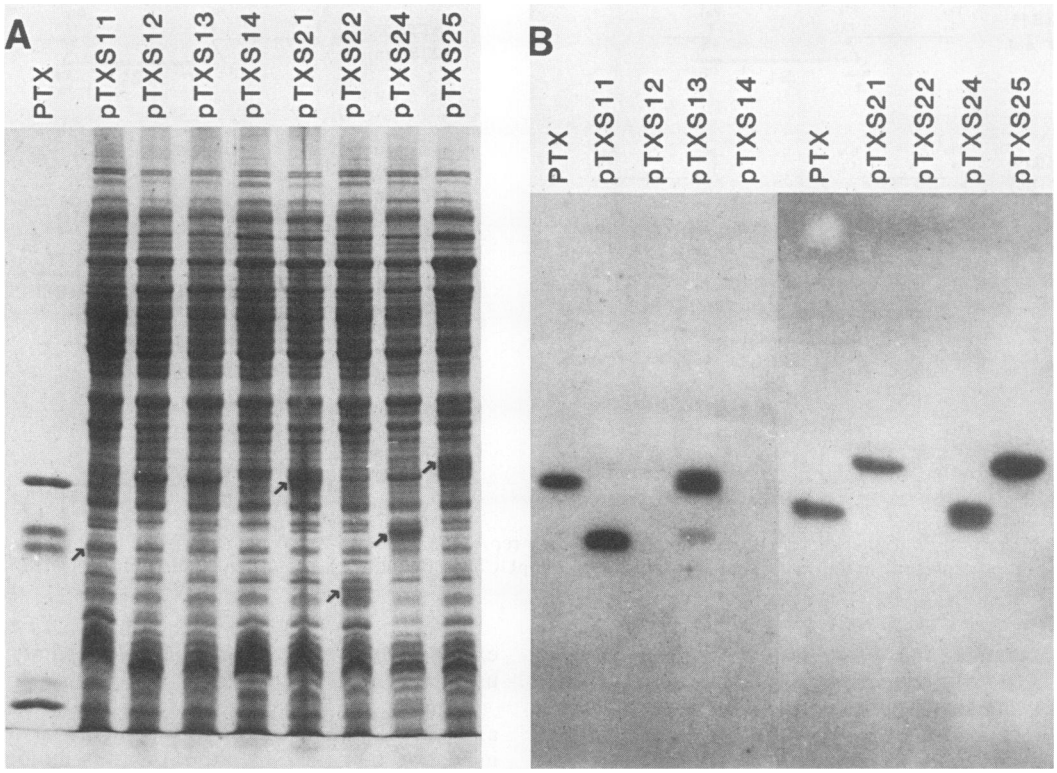

FIG. 3. Polyacrylamide gel electrophoresis and Western blot analyses of PTX subunit expression clones. Equal volumes of cells (1.5 ml) grown to saturation were pelleted by centrifugation and lysed in 150 μl of 1× Laemmli sample buffer. Samples (10 μl) were then electrophoresed in 12.5% gels. (A) SDS-PAGE of whole-cell lysates from *E. coli* containing the indicated plasmids. The gels were stained with Coomassie blue R-250. Arrows indicate the positions of induced protein bands. (B) Western blot of whole-cell lysates from *E. coli* containing the indicated expression vectors. Blots were incubated with either B2F8 anti-S1 (PTX, pTXS11, pTXS12, pTXS13, and pTXS14) or P11B10 anti-S2 (PTX, pTXS21, pTXS22, pTXS24, and pTXS25) monoclonal antibody.

cells containing either pTXS11 or pTXS13 also yielded a five- to sevenfold increase in the release of labeled nicotinamide from NAD (NAD-glycohydrolase activity) when compared with nontransformed *E. coli* lysates, pTXS24 extracts, or buffer alone (data not shown). In addition to showing that

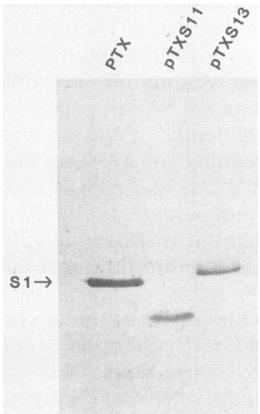

FIG. 4. Western blot analysis of *E. coli* lysates containing pTXS11 and pTXS13. *E. coli* cells containing either pTXS11 or pTXS13 were induced and subjected to French pressure cell disruption as described in Materials and Methods. Equal-volume amounts (25 μl) of the initial supernatant fraction of cells containing pTXS11 and the pellet fraction of cells containing pTXS13 were analyzed on Western blots with monoclonal antibody 1B7.

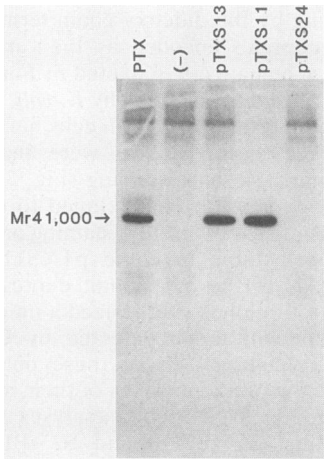

FIG. 5. ADP-ribosyltransferase activity of recombinant PTX subunits. CHO-K1 cell membranes (50 μg) were incubated with 1 μg of PTX, buffer (−), or 25-μl samples of recombinant subunit preparations from *E. coli* cells containing the indicated plasmids. The conditions are described in Materials and Methods. Reaction products were then analyzed by SDS-PAGE in a 12.5% gel followed by autoradiography. The arrow denotes the position of the M_r-41,000 band corresponding to the Gi protein.

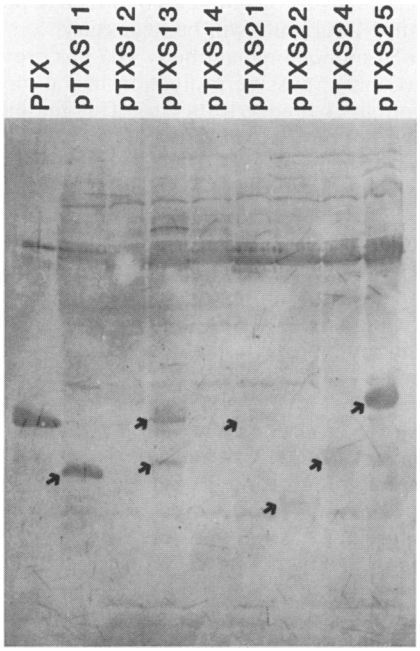

FIG. 6. Western blot analysis of recombinant PTX subunits with human hyperimmune serum. PTX and *E. coli*-whole cell lysates containing the indicated plasmids were analyzed on Western blots with human hyperimmune serum as described in Materials and Methods. Arrows denote bands at the positions expected for the recombinant proteins.

the recombinant S1 subunit is enzymatically active, these results demonstrate unambiguously that the S1 subunit of PTX is enzymatically active in the absence of the other subunits. This finding is in agreement with previous studies employing conventional biochemical separation techniques (11, 29).

Since the truncated version of S1 subunit encoded by pTXS11 retains enzymatic activity, the active sites for binding of both the acceptor and donor substrates as well as for catalysis must be located between amino acid residues 2 and 187. Furthermore, the first aspartic acid of the S1 subunit is not essential for enzymatic activity, since the recombinant S1 subunit lacks this residue.

Reaction of recombinant PTX subunits with human antiserum. Whole *E. coli* cell lysates containing the different versions of recombinant S1 and S2 subunits were analyzed by Western blotting with human hyperimmune serum. Purified PTX was included as a positive antigen control. Anti-PTX antibodies in human hyperimmune serum were detected by Western blot analysis (Fig. 6, lane 1). The S1 subunit yielded the strongest reactivity, indicating its immunogenicity in humans. Antibodies directed against S2 and S3 were also detectable upon close inspection and by comparison with Fig. 3. We were unable to detect anti-S4 or anti-S5 antibodies by this method. In other experiments with radiolabeled PTX, all subunits appeared to transfer with equal efficiency in the buffer system used (data not shown); therefore, differential transfer efficiencies among the subunits is not a contributing factor to the observed reactivity. As with natural PTX, the recombinant S1 subunits appeared to react more strongly with the human antiserum than the recombinant S2 subunits, except in the case of pTXS25. Irrespective of these observed differences in reactivity, this result demonstrates that the recombinant S1 and S2 subunits contain epitopes recognized by human antiserum raised against a whole-cell pertussis vaccine.

Immune response of mice to S2 subunit synthesized by *E. coli*. Since the recombinant subunits are recognized by both monoclonal antibodies and human hyperimmune serum, it was of interest to determine whether the subunits were immunogenic in experimental animals. Because of the routinely higher level of synthesis of S2 in *E. coli* when compared with that of S1 (Fig. 3A), we chose that subunit for preliminary immunization experiments.

Two groups of 10 CFW mice were immunized and boosted with either 150 or 300 μg of pTXS24 extract as described in Materials and Methods. Serum samples from three mice in each immunized group and from two unimmunized mice were analyzed for the presence of anti-PTX antibodies. Immunized mice from both groups produced increased amounts of anti-PTX antibodies (Table 1). Despite these increases, none of the sera at dilutions of 1/10 and greater inhibited PTX-mediated CHO cell clustering. When the mice were challenged by the intracranial route with live *B. pertussis*, no significant protection, as judged by survival, was observed among the mice immunized with the recombinant S2 subunit.

DISCUSSION

The S1 and S2 subunits of PTX can be synthesized as intracellular fusion proteins in *E. coli* when the expression of their respective genes is under *E. coli* transcriptional and translational control. By using pUC8 and pUC18 as expression vectors, the length of the vector-derived amino acid sequences was kept to a minimum and the signal peptides were removed. These results indicate that while the codon usage for these subunits is different from that of highly expressed genes in *E. coli*, this does not prevent their transcription and translation when placed in *E. coli*, as was previously conjectured (14).

The S1 subunit contains two cysteines, and chemical modification studies with sulfhydryl-reactive compounds and isolated S1 subunits suggest that one or both of them has a role in enzymatic activity (D. L. Burns, personal communication). In the truncated version of S1 (pTXS11), the cysteine proximal to the carboxy terminus of the molecule (Cys-199) has been deleted. Since this version retains both NAD-glycohydrolase and Gi:ADP-ribosyltransferase activi-

TABLE 1. Anti-PTX antibody response of mice immunized with S2 subunit expressed in *E. coli*

μg of S2 prepn injected and mouse no.	cpm[a]	
	Per mouse	Avg
0		
1	3,937	4,262
2	4,588	
150		
1	8,993	
2	11,301	9,179
3	7,224	
300		
1	10,888	
2	9,237	8,625
3	5,749	

[a] Counts per minute in radioimmunoassay with PTX-coated 96-well plates and ^{125}I-labeled rabbit anti-mouse IgG as the secondary antibody. Values given for individual mice represent the means of duplicate determinations.

ties Cys-199 is not essential for enzyme activity. These observations further suggest that Cys-41 is important for enzymatic activity. The assignment of a definitive role for Cys-41 in the catalytic mechanism of the S1 subunit is currently being pursued by oligonucleotide-directed mutagenesis and chemical modification of the recombinant S1 subunits. We have previously identified a Rossman-type structure containing the consensus sequence G-X-G-X-X-A between the first β-strand and the α-helix at the carboxy terminus of the S1 subunit (C. Locht, K. S. Marchitto, and J. M. Keith, Vaccines 87, in press). In nucleotide-binding proteins this structure is often implicated in binding of the phosphate moieties (18). Since the truncated S1 version does not contain this structure, it is not the NAD-binding site.

The truncated version of the S1 subunit (pTXS11) also appeared to be more soluble than either its full-length counterpart or any of the recombinant S2 molecules as judged by its presence in the initial supernatant after cell disruption. This increased solubility may be related to the lack of the carboxy-terminal residues (187 to 234) since this region appears to possess considerable hydrophobic character (data not shown). We are currently examining the possibility that, in addition to imparting solubility differences, this hydrophobic carboxy-terminal tail is also involved in the formation of stable associations between the S1 subunit and the remainder of the toxin molecule (B-oligomer).

After overnight induction, the cells containing pTXS13 also produced a second, smaller polypeptide that also reacted with monoclonal antibody B2F8 (Fig. 3) and with human hyperimmune serum (Fig. 5). This polypeptide migrated at the same position as a polypeptide (S1d) generated by either prolonged storage at 4°C or proteolysis of the native S1 subunit (24). Thus, it appears that the recombinant S1 subunit has a protease-sensitive site which is also present in the natural S1 subunit, thereby suggesting a conformational similarity between the two molecules.

The various truncated S1 and S2 subunit fusion proteins synthesized in *E. coli* can be used for mapping of other important regions, including epitopes that elicit protective antibodies. The clones expressing the S2 constructs allowed localization of the epitope for monoclonal antibody P11B10 to the 40-residue amino-terminal portion of the subunit. This antibody is specific for S2 and shows no cross-reaction with S3, despite the 70% overall amino acid homology between S2 and S3. These two subunits share less homology at their amino termini (14, 23), and analysis of the Hopp and Woods plot (7) in this area reveals the presence of two hydrophilic regions in S2, one of which is not found in S3 (data not shown). Since both of these hydrophilic regions are likely to constitute antigenic determinants, it follows that the P11B10 epitope may reside in the region spanning residues 22 to 29. Definitive mapping of this epitope and others, as well as target cell receptor-binding sites and S4 subunit-binding sites, is currently under investigation with various truncated versions of S2.

We were unable to map precisely the epitopes for anti-S1 monoclonal antibodies B2F8 and 1B7, owing to our inability to detect the smaller truncated versions of S1 in *E. coli*. Constructions of longer fusions to stabilize the truncated S1 versions encoded by pTXS12 and pTXS14 may be necessary to allow more precise mapping of the S1 epitopes and active sites. Alternatively, a protease-deficient *E. coli* mutant strain might be necessary for expression of these truncated S1 genes. Such genetic deletions in the S1 subunit gene may permit the production of an inactivated, yet immunologically potent form of S1. However, for active protection studies in mice, increased levels of synthesis or effective purification of recombinant S1 subunits will be necessary.

The anti-S1 monoclonal antibody 1B7 has previously been reported to confer passive protection in the mouse aerosol and intracranial challenge tests (26). This finding is consistent with the notion that the S1-associated enzyme activity plays a major role in the pathogenesis of whooping cough. A neutralizing anti-S2 monoclonal antibody has also been described (27). Our preliminary results indicate that the S2 subunit expressed in *E. coli* is able to elicit an anti-PTX immune response. However, we have not been able thus far to protect mice from intracranial challenge with *B. pertussis* by using recombinant S2 as the immunogen. Nevertheless, we are continuing protection studies using partially purified preparations of both recombinant S1 and S2 subunits.

In the present study we showed that PTX subunits can be synthesized by *E. coli*. The resultant recombinant proteins are enzymatically and antigenically very similar to the corresponding natural subunits. These findings illustrate the utility of the *E. coli* expression system in evaluating the ability of individual subunits to serve as protective antigens. This system, combined with such techniques as oligonucleotide-directed mutagenesis, will also allow detailed analyses of the relationship between structure and function of the various subunits. Studies on both the structure-function relationship and protective activities of recombinant subunits will greatly facilitate the development of a safer, new-generation vaccine against whooping cough.

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