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Cloning and sequencing of the pertussis toxin genes: Operon structure and gene duplication

(bacterial toxins/*Bordetella pertussis*/ADP-ribosylation/translational signals/secreted proteins)

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ABSTRACT **Pertussis toxin, a protein composed of five different subunits (S1, S2, S3, S4, and S5), is the major virulence factor of *Bordetella pertussis*. We have cloned and sequenced a DNA fragment of 4.7 kilobases that contains the genes coding for the five subunits. The genes are clustered within 3.2 kilobases in the following order: S1, S2, S4, S5, and S3. A sequence closely resembling *Escherichia coli* promoters is found only before the S1 gene, and a possible termination signal is present at the end of the S3 gene, which suggests that the pertussis toxin genes are organized in a single operon. A possible Shine–Dalgarno sequence is present before the S1 gene but not before the other four genes that 8–12 nucleotides upstream from the ATG codon show a new consensus sequence, 5′TCC(T)GG3′, possibly involved in the regulation of translation. We have also found sequence homology between the S2 and S3 genes and their protein products indicating that gene duplication played a major role in the evolution of pertussis toxin.**

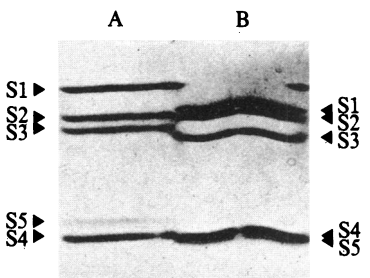

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of affinity-purified PT. The toxin in lane A has been treated with a reducing agent before loading the gel. The toxin in lane B has not been reduced. Note that although S2 and S3 have the same deduced molecular weight (Fig. 2), they have a different mobility in $NaDodSO_4$/PAGE. Note also that S5 is stained rather poorly and although its deduced molecular weight is smaller than that of S4 (Fig. 2) under reducing conditions, it migrates more slowly than S4.

Pertussis toxin (PT), a protein of 105 kDa that is released into the extracellular medium by virulent (phase 1) *Bordetella pertussis* (1–3), plays a major role in whooping cough (4, 5). Purified PT is composed of five different subunits (S1, S2, S3, S4, and S5) (Fig. 1). Like other bacterial protein toxins, such as cholera toxin, *Escherichia coli* heat-labile enterotoxin, diphtheria toxin, and pseudomonas exotoxin A (6, 7), PT can be divided into the following two functionally different moieties: A, which is the toxic subunit S1, and B (comprising S2, S3, S4, and S5), which is involved in binding the receptors on the surface of eukaryotic cells and in the translocation of the toxic subunit S1 across the cell membrane. S2, S3, and S4 are arranged in the following two dimers: D1 (S2 and S4) and D2 (S3 and S4), which are held together by S5 (3). Thus, PT contains a single copy of subunits S1, S2, S3, and S5 and two copies of subunit S4; it is in fact the most complex bacterial toxin described so far.

As described for diphtheria, cholera, and pseudomonas toxins, the toxic subunit A of PT is an NAD-dependent ADP-ribosyltransferase (6, 8). The enzyme substrates are the GTP-binding protein G_i, which is involved in the receptor-mediated inhibition of adenylate cyclase (9, 10), and transducin, which is involved in the translocation of signals from the light receptor rhodopsin to a cyclic GMP phosphodiesterase (11, 12). There is evidence that other GTP-binding proteins, also involved in the translocation of signals from membrane receptors to phospholipase C, are affected by PT (13–17). PT action causes irreversible uncoupling of the regulatory GTP-binding proteins from their membrane receptors and interferes with a variety of metabolic pathways having as secondary messengers cAMP and the breakdown products of phospholipids. The variety of names given to PT reflects this multiplicity of effects: islet-activating protein, histamine-sensitizing factor, and lymphocytosis-promoting factor. In addition, PT is also a mitogen and an adjuvant; it releases fatty acids from fat cells, interferes with chemotactic migration, changes the peripheral vascular permeability, and modifies the growth pattern of CHO cells *in vitro* (18–20).

PT is the major virulence factor of *B. pertussis* (4, 5), and it has been shown to induce protective levels of antibodies in animal models (20). A monoclonal antibody against PT was shown to protect mice from the challenge with virulent *B. pertussis* (21). Detoxified PT is a good candidate for a new acellular vaccine against pertussis (22). Manipulation of the toxin gene by genetic engineering could be a way to produce large amounts of detoxified protein. As nothing was known about the nature, structure, and expression of the PT gene(s), we have cloned and characterized it.

MATERIALS AND METHODS

Strains. *B. pertussis* strain BP165 was obtained from the Office of Biologics, Bethesda, MD (2). The Tn*5* mutant BP356 (23) was obtained from Stanley Falkow. *E. coli* strains JM101, Q359, and LE392 have been described (24, 25).

Amino-Terminal Sequencing of PT Subunits. PT from 50 liters of BP165 culture supernatant was affinity purified by Affi-Gel Blue and fetuin-Sepharose columns, as described by Sekura *et al.* (2). The five subunits were separated by preparative $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1) and electroeluted (26). The amino-terminal sequences

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Abbreviations: PT, pertussis toxin; bp, base pair(s); ORF, open reading frame.

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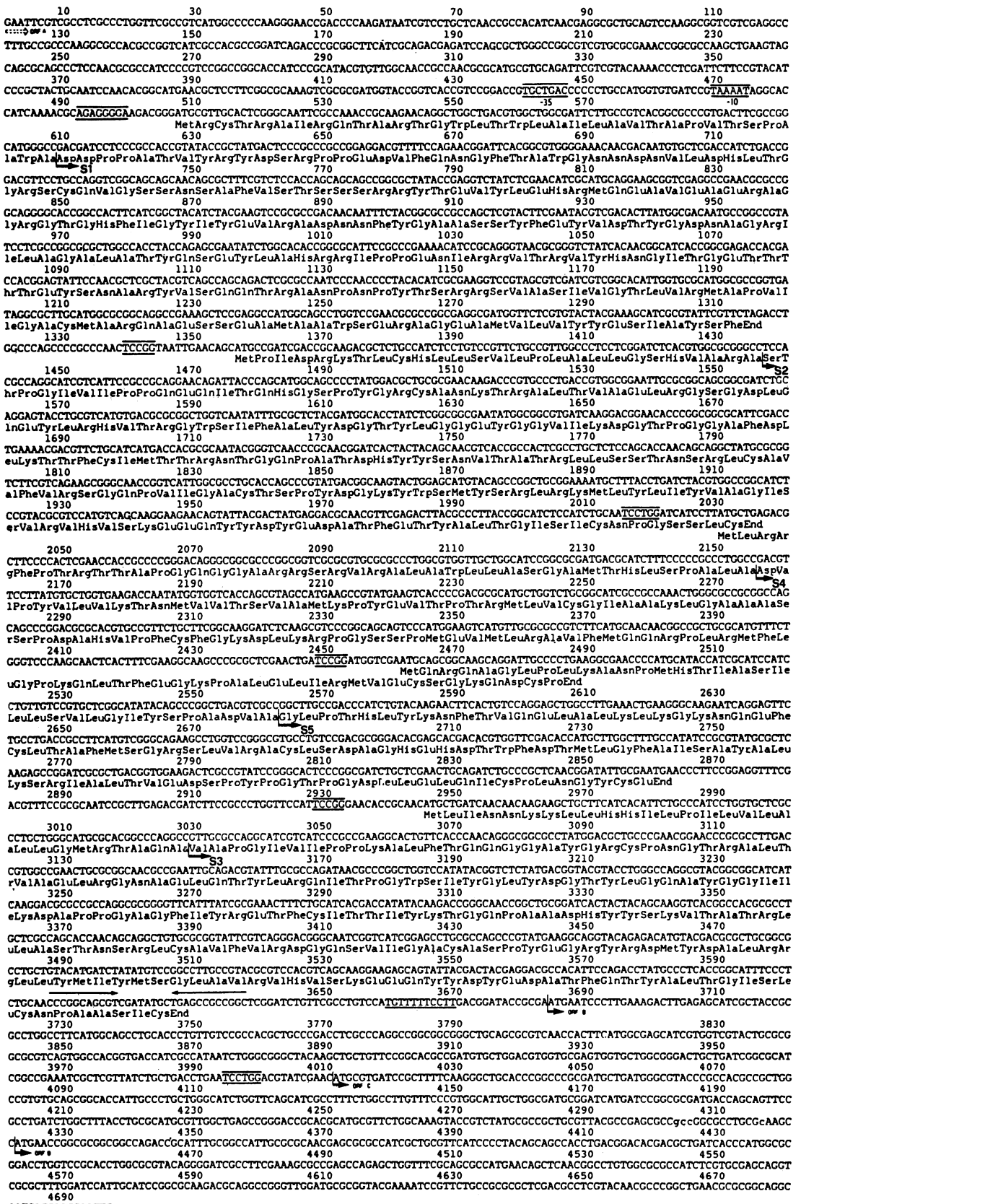

FIG. 2. Nucleotide sequence of the *Eco*RI fragment containing the five genes coding for PT. The deduced amino acid sequence of the five subunits of PT is reported. The arrows below the amino acid sequences indicate the beginning of the mature subunits, as identified by comparison with the amino-terminal sequences. In the case of S5, the arrow indicates the putative beginning of the mature subunit. Before the sequences of each subunit, we report the amino acid sequence of the putative signal peptides. Upstream from the gene coding for S1, the proposed promoter sequence and the putative Shine–Dalgarno sequences are boxed. The sequence TCC(T)GG before the S2, S3, S4, and S5 genes is also boxed. At the end of the gene coding for subunit S3, the arrows above the nucleotide sequence indicate the inverted repeat followed by the stretch of thymidines (underlined), which is a possible transcription termination site. The beginning of four ORFs having the same codon usage of the PT genes are indicated. The molecular weights deduced for the five subunits are the following: S1, 26,220; S2, 21,920; S3, 21,860; S4, 12,060; S5, 10,940.

were determined using the 470-A gas-phase microsequencer (Applied Biosystems, Foster City, CA).

Cloning and Sequencing. Chromosomal DNA was prepared from BP165 and BP356 using the procedure of Hull *et al.* (27). Genomic libraries were constructed in the phage vector EMBL4 (28) by partial *Sau*3A digestion (24). Amplification and screening of the library, restriction mapping, and Southern blot hybridization were performed by standard procedures (24). The *Eco*RI clone containing the toxin genes was digested from both ends with BAL-31 nuclease, subcloned in M13mp 18 and 19 (25) or pEMBL (29) vectors, and sequenced by the dideoxy chain-termination method (30). Computer analysis of data was performed using the software package of the University of Wisconsin Genetics Computer Group (Madison, WI).

RESULTS

Cloning Strategy. Following transposon mutagenesis, Weiss *et al.* (23) isolated a mutant of *B. pertussis* (BP356) that does not produce active toxin. This mutant had a single Tn*5* insertion in the chromosome, possibly located within the gene(s) coding for PT. If so, the nucleotide sequencing of the regions flanking the Tn*5* insertion should lead to the identification of the gene(s) coding for PT, provided that amino acid sequences of the proteins were available. We, therefore, purified PT, separated each subunit by preparative NaDodSO$_4$/PAGE (Fig. 1), and determined their amino-terminal sequences. The sequences obtained were the following: DDPPATVYRYDSRPPEDVFQNGFTAXG for S1; SQPGIVPPQEQITQHGSPY for S2; VAPGIVIPPKALFTQQGGAYGXXXNG for S3; DVPYVLVKTNMVVTSVAMKPYEVTP for S4. For reasons that are not clear, we were unable to obtain sequence data for the S5 polypeptide.

We constructed two genomic libraries in the λ phage EMBL4; one from the wild-type *B. pertussis* BP165 and the other from the Tn*5* mutant BP356. The latter was screened with a probe for Tn*5* DNA, and two positive clones were obtained from which we subcloned the fragments containing the junctions between the Tn*5* and the chromosomal DNA. The nucleotide sequence of the regions flanking Tn*5* contains an open reading frame (ORF) that was identified as coding for the amino-terminal region of the protein subunit S3, by comparison with the determined amino acid sequences. The Tn*5*-flanking sequences were then used as a probe to screen the phage library of the wild-type BP165 strain. Eight positive clones were obtained that contained a single *Eco*RI DNA fragment of 4.7 kilobases that could hybridize to the probe. This fragment was subcloned into the plasmid vector PEMBL8+ (29), and both strands were sequenced by progressive BAL-31 deletions from both ends of the fragment. The sequence of the 4696-base-pair (bp) *Eco*RI fragment is shown in Fig. 2.

Identification of the PT Genes. Computer analysis of the recombinant DNA sequence allowed the identification of several ORFs. A comparison of their coding properties with the sequence of the amino-terminal ends of the toxin subunits showed that four of the ORFs code for the protein subunits S1, S2, and S3, and S4 (Figs. 2 and 3). Molecular weight and amino acid composition of the deduced polypeptides are generally in good agreement with the published data (3). Although the amino-terminal sequence of the S5 polypeptide is not available, a fifth ORF, located between those coding for S4 and S3, codes for a protein with the expected molecular weight and amino acid composition of the protein subunit S5 (3).

FIG. 3. Schematic representation of the ORFs contained in the sequence shown in Fig. 2. Frames 1, 2, and 3 are shown from top to bottom; only the ORFs of at least 200 bp are reported. P, putative promoter sequence; T, putative terminator.

Structure of the Genes. The five ORFs are clustered within 3.2 kilobases in the following order: S1, S2, S4, S5, S3, and the ORF coding for S4 overlaps those coding for S2 and S5 (Figs. 2 and 3).

The amino terminus of each mature protein subunit is preceded by a peptide 27–42 amino acids long, containing the typical features of bacterial signal peptides (31, 32), indicating that the five subunits are translated as precursors. Since the signal peptides of subunits S1, S2, S3, and S4 end with the sequence (S)(P)AXA [in agreement with the consensus sequence AXA recognized by *E. coli* signal peptidases (33)], we can tentatively identify the beginning of subunit S5 (for which no amino-terminal sequence is available) after the sequence SPADVA.

The codon usage is the same in each of the five genes and is generally similar to that of *E. coli* (34). Among the other ORFs contained in the 4.7-kilobase fragment, four (ORF A, B, C, and D in Fig. 3) show the same codon usage and, therefore, they may encode unknown proteins.

Transcriptional and Translational Signals. The gene coding for S1 is preceded by a sequence that closely resembles the consensus sequence of the *E. coli* promoters (35, 36) (Fig. 4); a −10 region, TAAAAT that matches 5 of the 6 bp of the consensus sequence, is associated with a −35 region TGCTGACC that matches 6 of the 8 bp of the −35 consensus sequence. The distance between the proposed −35 and −10 regions is 21 bp. A possible ρ-independent terminator (36) can be identified at the end of the S3 gene (Fig. 5). Since no other promoter sequences can be found in the entire *Eco*RI fragment, it is likely that the five genes are organized in a single operon and are transcribed as a single polycistronic mRNA.

A sequence closely resembling the consensus Shine–Dalgarno sequence (37) is present 9 bp before the ATG of the S1 gene but not upstream from the other four genes which, however, contain the sequence TCC(T)GG 8–12 bp before each ATG start codon. The same sequence is also found 11 bp upstream from the first ATG of ORF C (Figs. 2 and 4).

Homology Between the S2 and S3 Genes. The genes coding for subunits S2 and S3 are 75% homologous within a region starting 19 bp before the ATG start codons (2 bp before the TCCGG sequences) and ending 5 bases after the TGA stop codons. The homology at the amino acid level between S2 and S3 is 67% (Fig. 6). This finding shows a common evolutionary origin for both sequences, possibly due to gene duplication.

DISCUSSION

Identification of the PT Genes. We have identified the structural genes for the five subunits of the *B. pertussis* toxin on the following grounds: (*i*) there is identity between the amino-terminal sequence of the proteins and the coding properties of the ORFs; (*ii*) the amino acid composition, molecular weight, and overall charge of the deduced polypeptides are consistent with the published data for each subunit; (*iii*) the ORF of each subunit encodes a signal peptide. The identification of the S5 gene provides additional evidence for the existence of this peptide, which has been controversial since in several laboratories the protein could not be visualized following NaDodSO$_4$/PAGE. We have also observed unusual properties of the S5 subunit, i.e., it is difficult to stain; under standard conditions, we were unable

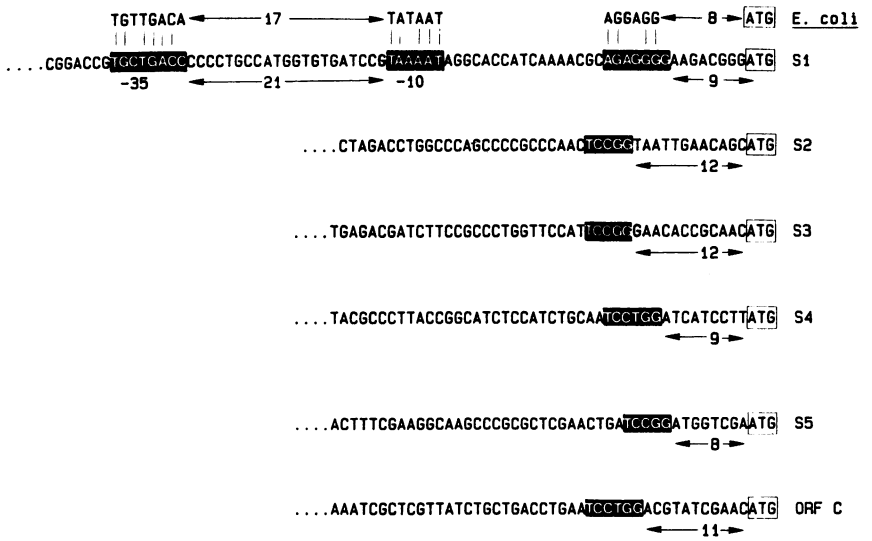

FIG. 4. Transcriptional and translational signals. The ATG start codons of the various ORFs have been aligned on the right (boxed). Upstream from the ATG of S1, the putative promoter and Shine–Dalgarno sequences are shown. The consensus *E. coli* sequences are reported above. Upstream from the ATGs of the remaining ORFs the sequence TCC(T)GG is shown. This sequence has not been found upstream from any other ATG codon in the entire nucleotide sequence reported in Fig. 2.

to sequence the amino terminus, and in spite of a deduced molecular weight of 10,900, its migration in $NaDodSO_4$/polyacrylamide gel is slower than that of the S4 subunit, which has a molecular weight of 12,000 (Fig. 1). Although post-translational modification(s) is unusual in bacteria, this could explain all these observations.

The *B. pertussis* Genes Are Organized as an Operon. The physical map of the five genes (Fig. 3) shows that they are not only clustered but three of them overlap each other. A transcriptional initiation signal very similar to the consensus sequence of *E. coli* promoters preceeds the cluster of genes while, at the 3′ end of the last gene of the cluster, we have identified an inverted repeat, which could form a stable stem and loop structure, followed by a stretch of thymidines. This may represent a ρ-independent termination site. Thus, the five PT genes are organized as a bacterial operon.

Transcriptional and Translational Signals. The analysis of the putative promoter shows that although the −10 and −35 sequence of *B. pertussis* are homologous to the *E. coli* consensus sequence, the distance between the two blocks is 21 bp instead of the optimal 17 bp reported for *E. coli*. Since such spacing has been shown to be very critical (35, 36), we could expect the PT promoter to be rather inefficient in *E. coli*. If the requirements for a good promoter are the same in *B. pertussis* and *E. coli*, this promoter should be inefficient also in *B. pertussis*, and the efficient expression of the gene, normally observed in phase I organisms, would imply some different regulatory mechanism. A positive regulatory element (*vir*) has actually been proposed to explain the phenomenon of phase variation in *B. pertussis* (38), i.e., the switch from the nonvirulent phase III to the virulent phase I, where PT and other virulence factors are expressed.

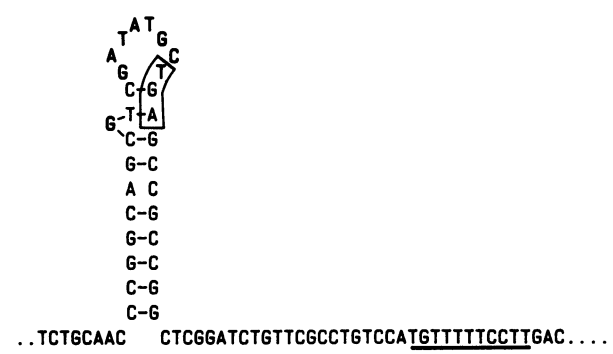

FIG. 5. Structure of the putative terminator.

The presence of a unique Shine–Dalgarno sequence upstream from the first gene of the operon strongly suggests that this is the ribosomal binding site that allows the translation of the S1 mRNA. The presence of the sequence TCC(T)GG in front of the remaining four genes, in a position corresponding to that of a ribosomal binding site, suggests that this sequence plays some role in the translation of the mRNA encoding the remaining subunits.

Secretion Signals. The presence of a signal peptide in each of the five subunits suggests that they are secreted individually into the periplasm. Although the structural features of the five signal sequences are those generally described for most signal peptides, the leader peptide of the S4 protein is unusually long (42 residues) and has the highest amino-terminal positive charge (+7) described so far (32). Since the positively charged amino-terminal region plays an important role in the efficiency of production of secreted proteins (39), the unusual structure of the S4 signal peptide may contribute

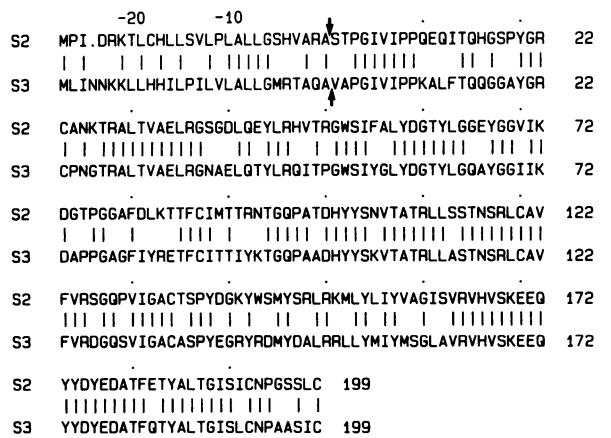

FIG. 6. Homology of amino acid sequences of subunits S2 and S3. The arrows indicate the cleavage site of the signal peptidase and beginning of the mature subunits.

FIG. 7. Homologies between the first 100 amino acid residues of the subunit S1 of PT (PT-S1) and the first 98 amino acid residues of cholera toxin fragment A (CT-A).

to a higher production of the S4 subunit that is present in a 2:1 stoichiometry in PT.

Particular Features of the PT Subunits. The S1 subunit is one of the few proteins that does not contain lysine residues. This observation has important implications for the development of a new vaccine against pertussis, since normally, for vaccine preparation, bacterial toxins are detoxified with chemicals that react mainly with lysine residues. Accordingly, we have observed that the detoxification of PT requires more severe conditions than those used for the other bacterial toxins and that, following treatment with glutaraldehyde, S2, S3, S4, and S5 are crosslinked and form aggregates of high molecular weight, while S1 retains its original size (data not shown).

The strong homology between the coding sequences and protein products of the S2 and S3 genes, while indicating a common ancestral origin, is not reflected by the functional properties of the two proteins. Tamura *et al.* (3) have in fact shown that in an *in vitro* system, the S2 and S3 proteins do not complement each other. Therefore, in this case two functionally distinct proteins have evolved from the duplication of one gene.

Evolutionary Considerations. We have compared the nucleotide and amino acid sequence of the PT genes and proteins with those of other bacterial protein toxins having similar ADP-ribosylating activity.

Although the nucleotide sequences are completely different, the amino acid sequences of the fragment A of cholera toxin (40) and the S1 subunit of PT contain homologous amino acid residues clustered at the amino-terminal end of the two proteins (Fig. 7), which use the same NAD substrate for the ADP-ribosylation of different protein targets. This suggests that the homologous regions of the two proteins may be those interacting with NAD.

The structure of the PT operon could be described as a central core containing the S2, S3, and S4 overlapping genes, flanked by the S1 and S3 genes. Such organization suggests a model for the evolution of the operon where the S3 gene is derived from the initial core structure, by duplication of the S2 gene. The S1 gene, as suggested by Pappenheimer (41), could have been acquired by the fusion of a eukaryotic ADP-ribosylase gene to the other four genes.

We wish to thank Stanley Falkow for supplying the Tn*5* mutants of *B. pertussis* and for communicating unpublished data on their properties. Particular thanks are due to Claudio Basilico for advising us to approach the study of *B. pertussis*, and to Marialuisa Melli for useful advice and for critically reading the manuscript. We also thank Giuseppe Corsi for valuable technical help, Francesco Di Pisa for assistance with the computer, and Giorgio Corsi for graphic work.

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