Further Analysis of the Sequence of the S1 Subunit of Pertussis Toxin

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The two published sequences of the pertussis toxin operon differ in 3 bp in the S1 subunit gene. In this report, we provide evidence that Bordetella pertussis strains are able to produce active pertussis toxin only when they contain one of the two possible nucleotide sequences.

Pertussis toxin (PT) is an oligomeric protein released into the culture medium by Bordetella pertussis, the human pathogen responsible for whooping cough (18, 21). This protein, which is a major virulence factor and the main component of new acellular vaccines against whooping cough (2, 14, 16, 17, 19, 22), contains five noncovalently linked subunits (S1, S2, S3, S4, and S5) which are assembled into the A monomer (subunit S1) and the B oligomer (containing subunits S2, S3, S4, and S5, present in a 1:1:2:1 ratio) (18, 21). Each of the five subunits is synthesized as a precursor containing a secretory leader peptide and is secreted into the periplasm of B. pertussis, where toxin assembly takes place. The assembled holotoxin is eventually secreted into the culture medium. We have recently shown that strains of B. pertussis which produce S1 subunits with altered conformation are unable to secrete the holotoxin and secrete into the medium only very low amounts of the B oligomer of pertussis toxin (13).

The genes coding for the five PT subunits are contained in a 4.7-kb EcoRI fragment and are organized as an operon (7, 9). The PT operon has been cloned from two different B. pertussis strains, and its nucleotide sequence has been determined independently by two groups (7, 9). Remarkably, the two nucleotide sequences showed only a 3-bp difference in the S1 subunit gene. The nucleotides G-1184, C-1185, and G-1203 of the sequence described by Nicosia et al. (9) are not present in the sequence of Locht and Keith (7). This results in the deletion of one amino acid and seven amino acid substitutions in the 193 to 200 region of the S1 protein (Fig. 1). The difference in amino acid sequence, although minimal, has already caused some ambiguities, especially in those studies in which synthetic peptides were used to investigate B- and T-cell epitopes (11, 12). So far, both sequences have been considered possible and no efforts have been made to elucidate this discrepancy.

During physicochemical characterization of the S1 subunit of PT, we performed a mass spectrometric study of this subunit. The tryptic digest of S1 was directly analyzed by fast atom bombardment (FAB) mass spectrometry, following the FAB mapping strategy (8). The FAB mass spectra were recorded with a VG ZAB 2SE mass spectrometer equipped with a cesium gun operating at 25 KeV (2 μA). The tryptic digest was lyophilized and dissolved in 5% acetic acid. A 2-μl sample was loaded onto a glycerol-coated probe tip.

Spectra were recorded on UV paper and manually counted; signals were assigned to the corresponding peptides along the S1 sequence by using a computer program designed for this purpose (15). Several signals corresponding to expected tryptic peptides were recorded, so that it was possible to confirm a great deal of the entire S1 sequence (Fig. 2). A strong signal at m/z 1101 was assigned to peptide 183 to 193, which contained an arginine residue at the C terminus. The presence of Arg-193 was inferred on the basis of the molecular weight of peptide 183 to 193 and the specificity of the protease used. The occurrence of a tryptic hydrolysis site at position 193 resulted in the formation of an additional peptide 11 residues long which gave rise to a signal at m/z 1119 which matched the molecular weight of the fragment 194 to 204 in the amino acid sequence of Nicosia and Rappuoli (9). These results clearly show that amino acid 193 is an Arg, according to Nicosia and Rappuoli (9), and not His as determined by Locht and Keith (7).

To investigate whether there are two different genes coding for the S1 subunit or whether the difference in the published sequence is due to a mistake, we performed site-directed mutagenesis (14) on the S1 subunit derived from the clone sequenced by Nicosia and Rappuoli (9) to obtain an S1 subunit having the sequence published by Locht and Keith (7). The mutagenesis was done with the 0.9-kb Kpn-Xba fragment which encodes the S1 subunit (7, 9). The BlueScript KS plasmid (Stratagene, San Diego, Calif.) was used as a vector to prepare single-stranded DNA. First, we used the oligonucleotide primer 1193-5' CCGGT GATAGCGCTTGCA 3'-1213 to introduce the deletion of

![Comparison of the two nucleotide and amino acid sequences of S1 a, a', Nucleotide and amino acid sequences according to Nicosia et al. (9). b, b', Nucleotide and amino acid sequences according to Locht and Keith (7).](http://iai.asm.org/)

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FIG. 2. FAB mass spectrum of the tryptic digest of electroeluted S1 subunit. SDS electrophoresis was performed by the method of Laemmli (6), and the S1 band was electroeluted and released from SDS by the procedure described by Henderson et al. (3). A sample of about 0.4 mg was digested with trypsin (1:50 [wt/wt]; TPCK [tolylsulfonyl phenylalanyl chloromethyl ketone] treated trypsin purchased from Sigma Chemical Co., St. Louis, Mo.) in 0.4% ammonium bicarbonate (pH 8.5) for 4 h at 37°C. The peaks which were unequivocally assigned by the computer program along the S1 sequence are reported in the figure. Each peak reports the molecular mass and the coordinates of the S1 peptide.

FIG. 3. Polyacrylamide gel showing the nucleotide sequence of the S1 subunit gene after site-directed mutagenesis to make its sequence identical to the one published by Locht and Keith (7). The arrows indicate the expected positions of the nucleotides which have been deleted.
subunits was determined by Western immunoblotting. Wild-type *B. pertussis* cultures were grown and processed in parallel as a control. Monoclonal antibody 6G7 was used to detect the S1 subunit, and monoclonal antibodies 4B8 and 4D10 were used to detect the S4 and S5 subunits (1).

By Western immunoblotting, we could detect low amounts of S4 and S5 subunits but not traces of the S1 subunit in the culture supernatant of BP 10001 (Fig. 4). By enzyme-linked immunosorbent assay (10), BP 10001 produced approximately ten times less PT than the wild-type strain. No toxic activity could be detected in the CHO cell assay (4).

The data obtained with BP 10001 are identical to those obtained with mutants BP-8D9G, BP-50E, BP-88E/89S and BP-13L/261/129G, which produce S1 subunits with altered conformation and secrete into the culture medium only low amounts of the B oligomer (13). We conclude that a *B. pertussis* strain having an S1 subunit with the sequence of Locht and Keith (7) does not secrete an active PT into the culture medium. Subunits S4 and S5 can be detected in the medium; subunit S1 cannot. By analogy with the other mutants which behave similarly and secrete only the B oligomer (13), we propose that the 3-bp changes in BP 10001 result in an S1 subunit with altered properties and/or conformation which cannot be assembled into the holotoxin. Under these conditions, only a low amount of the B oligomer is secreted. Since mutant BP 10001 is not able to produce PT, the discrepancy in nucleotide sequence is likely to reflect a sequencing error and not a heterogeneity of the PT genes.

REFERENCES