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Molecular cloning of pertussis toxin genes

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ABSTRACT

We have cloned a 4.5 kb EcoRI/BamHI DNA fragment from Bordetella pertussis which contains at least two genes responsible for expression of pertussis toxin. The S4 subunit of the toxin was isolated by high pressure liquid chromatography and the NH_2-terminal amino acid sequence determined. Using a mixed synthetic oligonucleotide probe designed by reverse translation of a portion of the protein sequence, a cloned DNA fragment was identified which contains the coding information for at least the S4 structural subunit of the toxin. Sequence analyses indicate that the mature protein is derived by proteolytic cleavage of a precursor molecule. Southern blot analyses of Tn5-induced B. pertussis toxin-deficient mutants show that the Tn5 DNA is inserted 1.3 kb downstream from the S4 subunit gene. This second gene could code for another subunit required for assembly of the mature toxin or a non-structural transport protein, possibly in the same polycistronic operon. The molecular cloning of pertussis toxin genes provides the basis for development of a safer recombinant "new generation" vaccine for whooping cough.

INTRODUCTION

Development of a safer, "new generation" vaccine for whooping cough can be accomplished by molecular cloning and genetic manipulation of the genomic DNA of B. pertussis, the microorganism responsible for the disease. One important antigenic component of the current vaccine is pertussis toxin, also known as lymphocytosis promotion factor, histamine-sensitizing factor, islet-activating protein, and pertussigen (1). It is one of several toxins produced by virulent B. pertussis (2). While this toxin is one of the major protective antigens against whooping cough, as shown by intracerebral (3,4) or aerosol (4) challenge in mice, and is used in the component vaccine in Japan (5) it is also associated with a variety of pathophysiological activities and has been suggested to be the major cause of the harmful side effects associated with the present pertussis vaccine. In most recipients these effects are limited to local reactions, but regrettably, in rare cases neurological damage and sometimes death occurs (see ref. 6 for review and references).

Pertussis toxin is a globular complex protein composed of five different

subunits. The 117,000 molecular weight native toxin can be separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) into individual subunits with molecular weights of 28 kD (S1), 23 kD (S2), 22 kD (S3), 11.7 kD (S4), and 9.3 kD (S5) (see Fig. 1); the molar ratio of the subunits is 1:1:1:2:1, respectively (7). By analogy to diphtheria toxin, Pseudomonas toxin, cholera toxin, and Escherichia coli heat-labile toxin, pertussis toxin is structured in an A-B model (8). In this model, the A-moiety (S1 in pertussis toxin) contains an enzymatic activity responsible for the ADP-ribosylation (9) of specific regulatory proteins. The B-moiety (S2 through S5 in pertussis toxin) is responsible for attachment of the toxin to the host cell membrane and internalization of the A-protomer into the target cell (10). Once inside the host cell, S1 (dissociated from the B-oligomer) catalyzes the transfer of the ADP-ribosyl moiety of NAD^+ to the membrane bound guanyl nucleotide-binding regulatory protein (Ni or Gi) of the adenylate cyclase complex (11,12). This covalent modification of Ni results in an uncoupling of Ni from inhibition receptors. Thus, activation of the adenylate cyclase catalytic subunit by stimulatory receptors cannot be reversed by inhibitors, which subsequently results in an increase of intracellular cAMP (13). This increase in cAMP may be the cause of many of the biological effects associated with pertussis toxin.

Progress in the development of a cloned subunit vaccine has been hampered by the inherent difficulties in identifying DNA fragments containing specific genes which code for protective antigens. The polycistronic gene arrangement of procaryote cells, similarities with other bacterial toxins (14-20), and preliminary mutagenic experiments by other investigators (21) suggest that the genes coding for the protein subunits of pertussis toxin may be linked in a tandem arrangement regulated by one operator. Thus, isolation of a DNA fragment containing one subunit gene could quickly lead to the isolation and characterization of all the genes responsible for pertussis toxin. Recent pertussis DNA studies in our laboratory using recombinant expression vectors and pertussis toxin antibodies suggest that cloning strategies which rely on expression of protein in an E. coli host have not been successful, probably because of differences in the signals that regulate gene transcription. A more direct approach using synthetic oligonucleotide probes which code for specific portions of antigenic proteins has proven to be more effective in identifying cloned DNA fragments of interest. We describe here the molecular cloning of pertussis toxin genes in E. coli, a first step in the development of a safer pertussis vaccine.

MATERIALS AND METHODS

Purification of Pertussis Toxin Subunits.

Pertussis toxin from strain 3779 was prepared as previously described (22). Five mg of the toxin was resuspended in trifluoroacetic acid and fractionated by HPLC using a 1 X 25 cm Vydac C-4 preparative column. The sample was injected in 50% trifluoroacetic acid and eluted at 4 ml/min over 30 min with a linear gradient of 25% to 100% acetonitrile solution containing 66% acetonitrile and 33% isopropyl alcohol. All solutions contained 0.1% trifluoroacetic acid. Elution was monitored at 220 nm and two ml fractions collected. Aliquots of selected fractions were dried by evaporation, resuspended in gel loading buffer containing 2-mercaptoethanol and analyzed by SDS-PAGE on a 12% gel.

Protein and DNA Sequencing.

The polypeptide from HPLC fraction 21 (see Fig. 1, lane 4) was sequenced using a Beckman 890C automated protein sequenator. The methods have been described previously (23). DNA was sequenced from the SmaI site (see Fig. 2b) by the Maxam and Gilbert technique (24).

Isolation of Pertussis Toxin Genes.

Chromosomal DNA was prepared from B. pertussis strain 3779 as previously described (25). The DNA was digested with both endonucleases EcoRI and BamHI and ligated into the same sites in the polylinker of pMC1403 (26,27). The conditions for ligation were: 60 ng of vector DNA and 40 ng of insert DNA incubated with 1.5 units of T_4 DNA ligase (BRL) and 1 mM ATP at 15°C for 20 h. E. coli JM109 cells were transformed with the recombinant plasmid (28) and clones containing the toxin gene identified by colony hybridization at 37°C (29) using a ^{32}P-labeled 17-base mixed oligonucleotide probe 21D3. The probe was synthesized on a SAM-1 DNA synthesizer (Biosearch, San Rafael, California) and consisted of the 32 possible oligonucleotides coding for 6 consecutive amino acids of the pertussis toxin subunit (see Table 1). The probe was purified from a 20% urea-acrylamide gel and 5'-end labeled using 0.2 mCi of (gamma^{32}P)ATP (ICN, crude, 7000 Ci/mmol) and 1 unit of T_4 polynucleotide kinase (BRL) per 10 µl of reaction mixture in 50 mM Tris-HCl (pH 7.4), 5 mM DTT, 10 mM $MgCl_2$. The labeled oligonucleotides were purified by binding to a DEAE-cellulose column (DE52, Whatman) in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA (TE) and eluted with 1.0 M NaCl in TE. Ten positive clones were isolated and purified. Plasmid DNA from these clones was extracted (27), digested with a variety of restriction endonucleases (BRL), and then analyzed by 0.8% agarose gel electrophoresis in TBE (10 mM Tris-borate pH 8.0, 1 mM EDTA). Southern

blot analysis using the ^{32}P-labeled oligonucleotide 21D3 as the probe showed that all 10 clones contained an identical insert of B. pertussis DNA. One clone was used for further analysis by Southern blots (described in Fig. 3) and DNA sequencing (24).

Southern Blot Analyses.

DNA was extracted as previously described (25). Digested DNA was separated by electrophoresis using either 0.7% or 1.2% agarose gels in 40 mM Tris-acetate pH 8.3, 1 mM EDTA for 17 h at 30 V. The DNA was then blotted onto nitrocellulose in 20X SSPE (27) and baked at 80°C in a vacuum oven for 2 h. Filters were prehybridized at 68°C for 4 h in 6X SSPE, 0.5% SDS, 5X modified Denhardt's (0.1% Ficoll 400, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone and 0.3X SSPE) and 100 µg/ml denatured herring sperm DNA. The hybridization buffer was the same as the prehybridization buffer, except EDTA was added to a final concentration of 10 mM. PstI fragments A, B, C and D were isolated by 0.8% low-melting point agarose gel electrophoresis, purified on Elutip-d columns (Schleicher and Schuell) and nick translated (BRL) using (alpha^{32}P)CTP (800 Ci/mmol, NEN Research Products). The nick translated probes were hybridized at a concentration of about 1 µCi/ml for 48 h at 68°C. Filters were then washed in 2X SSPE and 0.5% SDS at room temperature for 5 min, then in 2X SSPE and 0.1% SDS at room temperature for 15 min, and finally in 0.1X SSPE and 0.5% SDS at 68°C for 2 h. The washed filters were air dried and exposed to X-ray film using a Lightning-Plus intensifying screen.

RESULTS AND DISCUSSION

Isolation and cloning of S4 subunit gene.

Purified pertussis toxin (22) from B. pertussis strain 3779 was fractionated by high pressure liquid chromatography (HPLC). One fraction (Fr21) contained a polypeptide which comigrated as a major band with subunit S4 on SDS-PAGE (Fig. 1, lane 4). Although complete separation was not achieved, the major portion of the other toxin subunits were recovered in other HPLC fractions, i.e., S2 in Fr22, S1 and S5 in Fr23, and S3 in Fr24 (Fig. 1). The amino acid sequence of the first 30 NH_2-terminal residues of the protein in fraction 21 was determined and is shown in Table 1. Based on this protein sequence data we synthesized a mixed oligonucleotide probe representing a region of six consecutive amino acids with the least redundancy of the genetic code. In this mixture of oligonucleotides, identified as probe 21D3, approximately 1 out of 32 molecules should correspond to the actual DNA sequence of the pertussis toxin gene (Table 1). This mixed oligonucleotide

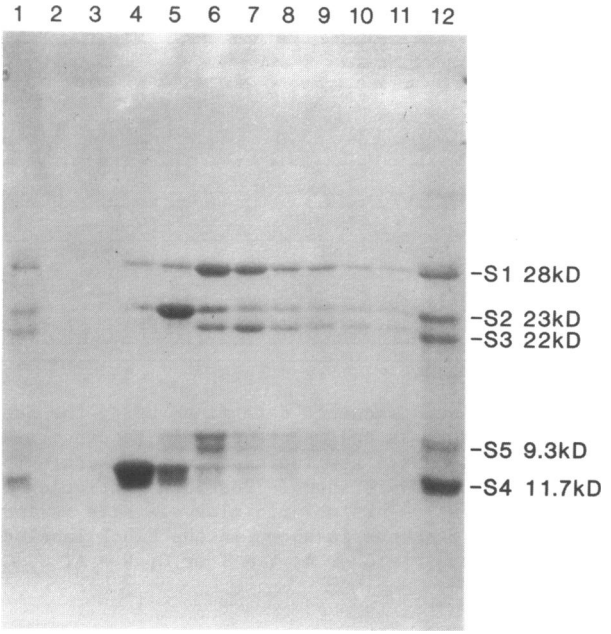


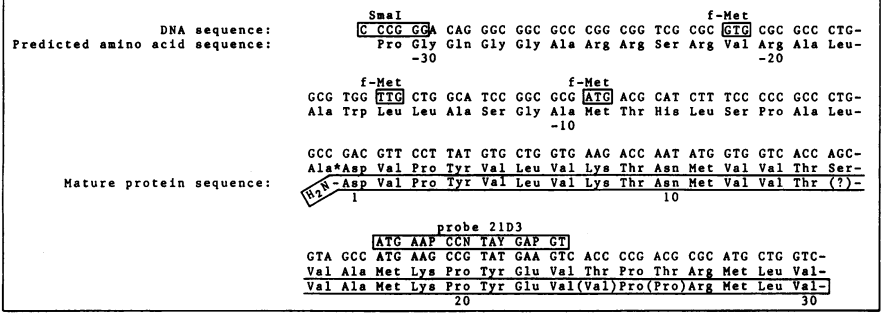
Fig. 1. SDS-electrophoresis of the products of HPLC separation of pertussis toxin. Lanes 1 and 12 contain 5 µg and 10 µg, respectively, of unfractionated pertussis toxin. Lanes 2 through 11 contain 100 µl aliquots of elution fractions 19 through 28, respectively. The molecular weights of the subunits are indicated.

probe was used to screen a DNA clone bank containing restriction fragments of total pertussis chromosomal DNA. The clone bank was prepared by digesting genomic DNA isolated from B. pertussis strain 3779 with both EcoRI and BamHI restriction endonucleases. The complete population of restriction fragments was ligated into the EcoRI/BamHI restriction site of expression vector pMC1403 (26) and the recombinant plasmid used to transform E. coli JM109 cells. Approximately 20,000 colonies were screened by colony hybridization using the ^{32}P-end labeled oligonucleotide probe 21D3. The plasmid DNA of 10 positive colonies was examined by restriction enzyme and Southern blot analyses. All 10 colonies contained a recombinant plasmid with an identical 4.5 kb EcoRI/BamHI pertussis DNA insert. One of these clones, identified as pPTX42, was selected for further characterization. A restriction map of the insert DNA was prepared and is shown in Figure 2b; Southern blot analysis indicated that the oligonucleotide probe 21D3 hybridized to only the 0.8 kb SmaI/PstI fragment.

Sequencing of the H_2N-terminal region for S4.

This 0.8 kb fragment was isolated by agarose gel electrophoresis and

Table 1. Protein and DNA Sequences of Pertussis Toxin Subunit, Oligonucleotide Probe and Homologous Genomic DNA Clone



The S4 H_2N-terminal amino acid sequence determined using the automated protein sequenator is shown in blocks as the mature protein sequence. Residues that were questionable in the sequence are indicated by brackets. The DNA and predicted amino acid sequences are shown. Possible initiation codons are indicated by f-Met. A putative proteolytic cleavage site is indicated by *. The oligonucleotide probe sequence is shown in the block labeled probe 21D3. The abbreviations used are: P = G or A; Y = T or C; N = A, C, G or T.

sequenced using the Maxam and Gilbert technique (24). The DNA sequence was translated into an amino acid sequence and a portion of that sequence is compared in Table 1 to the NH_2-terminal 30 amino acids of the pertussis toxin subunit and the oligonucleotide probe 21D3 sequence. Out of the sequence of 30 amino acid residues determined using the automated sequenator, only 2 do not correspond to the amino acid sequence deduced from the DNA sequence, i.e., residues 24 and 26 are questionable because they repeat the amino acid in front of them and they are located near the end of the analyzed sequence. Amino acid 15 could not be determined. The rest of the deduced amino acid sequence perfectly matches the original protein sequence. The oligonucleotide probe sequence also perfectly matches the cloned DNA sequence. These results indicate that we have cloned at least one of the pertussis toxin subunit genes.

Examination of the DNA sequence indicates that a precursor protein, perhaps containing a leader sequence, may exist (Table 1). In fact, the NH_2-terminal aspartic acid of the mature protein is not immediately preceded by one of the known initiation codons, i.e., ATG, GTG, TTG, or ATT (30), but by GCC coding for alanine, an amino acid that often occurs at the cleavage site of a signal peptide (31-33). A proline is found at amino acid position -4, which is also consistent with cleavage sites in other known sequences where this amino acid is usually present within six residues of the cleavage site. Possible translation initiation sites in the same reading frame as the mature

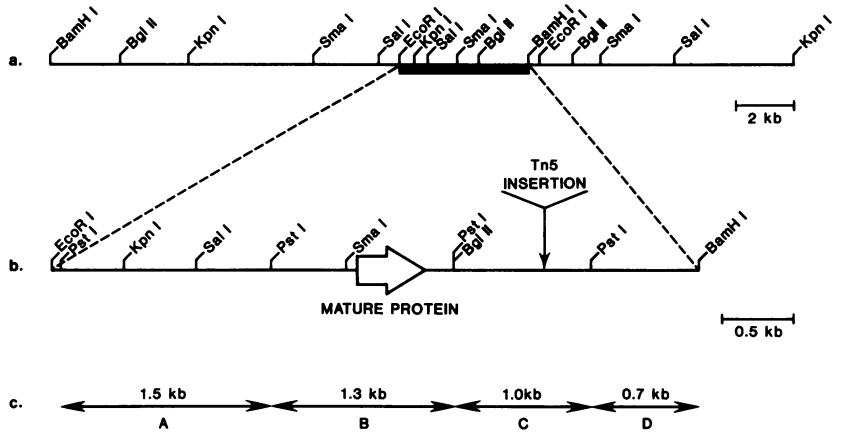


Fig. 2. Restriction map of the cloned 4.5 kb EcoRI/BamHI B. pertussis DNA fragment and genomic DNA in the region of the pertussis toxin subunit gene. (a) Restriction map of a 26 kb region of B. pertussis genomic DNA containing pertussis toxin genes. (b) Restriction map of the 4.5 kb EcoRI/BamHI insert from pPTX42. The arrow indicates the start and translation direction of the mature toxin subunit. The location of the Tn5 DNA insertion in mutant strains BP356 and BP357 is shown. (c) PstI fragment derived from the insert shown in panel b.

protein and upstream of the NH_2-terminal aspartic acid are: ATG at position -9, TTG at -15, and GTG at -21; however, none of these are preceded by a Shine/Dalgarno ribosomal binding site (34) and only GTG at -21 is immediately followed by a basic amino acid (arginine) preceding a hydrophobic region, characteristic of bacterial signal sequences (35,36). The unusual GTG translation initiation codon is used in the diphtheria toxin gene (14). Using the DNA sequence data and primer extension to sequence the mRNA we may be able to determine the actual initiation site.

Physical mapping of the S4 gene on the bacterial chromosome.

We then used the 1.3 kb PstI fragment B containing at least part of the pertussis toxin gene as a probe to physically map the location of this gene on the B. pertussis genome (see Fig. 2). Figure 3a shows a Southern blot analysis of total B. pertussis DNA digested with a variety of six base pair-specific restriction enzymes and probed with the 1.3 kb PstI fragment B isolated from pPTX42. Each restriction digest yielded only one DNA band which hybridized with the probe. Since the 1.3 kb PstI fragment B contains a SmaI site, two bands would be expected from a SmaI digest of genomic DNA unless the SmaI fragments were similar in size. Further analysis indicated that the single

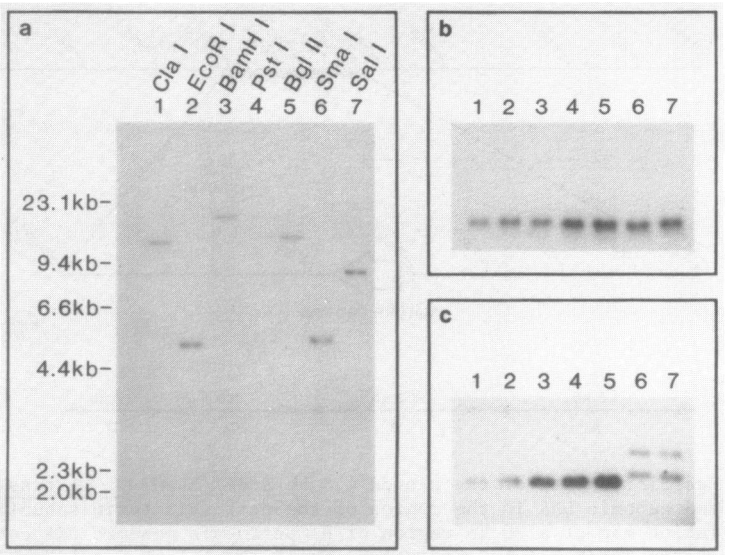


Fig. 3. Southern blot analysis of B. pertussis genomic DNA with cloned DNA probes. (a) Total genomic DNA from strain 3779 was digested with various restriction enzymes as indicated on the figure, and analyzed by Southern blot using nick translated PstI fragment B of pPTX42 (see Fig. 2c). (b) Between 24 µg and 60 µg of genomic DNA from strains 3779, Sakairi^{+} (pertussis toxin^{-}, Tn5^{-}), BP347 (non-virulent, Tn5^{+}), BP349 (hemolysin^{-}, Tn5^{+}), BP353^{+} (filamentous hemagglutinin^{-}, Tn5^{+}), BP356 and BP357 (both pertussis toxin^{-}, Tn5^{+}) (15) (lanes 1 through 7, respectively) were digested with PstI and analyzed by Southern blot using nick translated PstI fragment B as the probe. (c) The same as panel b except PstI fragment C was used as the probe.

band seen in the SmaI digest is actually a doublet of two similar size DNA fragments. In this particular gel, fragments of 1.3 kb and smaller migrated off the gel during electrophoresis and thus could not be detected; however, in other Southern blots in which no fragment was run off the gel, only one band was found for each restriction enzyme. These results indicate that the gene encoded by the PstI fragment B occurs only once in the genome. Using the data from these experiments and similar studies using the 1.5 kb PstI fragment A and the 0.7 kb PstI/BamHI fragment D from the cloned 4.5 kb EcoRI/BamHI fragment, we prepared a partial restriction map of a 26 kb region of the pertussis genome as shown in Figure 2a. This method allowed us to locate the first restriction site of a particular endonuclease on either side of the 4.5 kb EcoRI/BamHI fragment. This information will be useful in understanding the genetic arrangement of the toxin genes and for the subsequent cloning of larger and

possibly more complete DNA fragments, perhaps containing genetic information for expression or regulation of pertussis toxin.

Relationship of the S4 gene and Tn5-insertions.

Weiss and her colleagues have developed several important Tn5-induced B. pertussis mutants deficient in different virulence factors, i.e., pertussis toxin, hemolysin, and filamentous hemagglutinin (21,37,38). To investigate the physical relationship between the Tn5 DNA insertion and the pertussis toxin subunit gene, we analyzed genomic DNA from these mutants and strain 3779 by Southern blots using various restriction fragments of the cloned 4.5 kb EcoRI/BamHI DNA fragment as probes. In one set of experiments, blots of genomic PstI fragments were separately probed with cloned PstI fragments A, B, C, and D (see Fig. 2c). The PstI fragments from the mutants and strain 3779 which hybridized with the cloned PstI fragments A, B, and D were exactly the same size; the blot probed with PstI fragment B is shown in Figure 3b. However, when the PstI fragment C was used as a probe, the genomic DNA from mutant strains BP356 and BP357 showed a clear difference in the size of the PstI fragments that hybridized as compared to strain 3779 and the other mutant strains (Fig. 3c, lanes 6 and 7). These results indicate that this fragment contains the site of the Tn5 insertion. As expected, two labeled fragments were found, since the Tn5 DNA insert has two symmetrical PstI sites. Other Southern blots (not shown) in which genomic BglII and SmaI fragments were hybridized with the 4.5 kb EcoRI/BamHI cloned probe, and the data from Figure 3c, clearly show that the Tn5 DNA was inserted 1.3 kb downstream from the start of the mature pertussis toxin S4 subunit in the two mutant strains that were characterized as pertussis toxin negative phenotypes, i.e., BP356 and BP357 (see Fig. 2b). This insertion is beyond the termination codon for the S4 subunit (11.7 kD). Examination of these toxin negative mutants by Western blots using monoclonal antibodies for individual subunits indicate that the Tn5 DNA is not inserted in the subunit structural genes for S1 or S2 (unpublished results). The pertussis toxin negative phenotype of strains BP356 and BP357 can be explained by either of two nonexclusive mechanisms. The Tn5 DNA may be inserted into the coding regions of either S3, S5, or perhaps another gene required for toxin assembly or transport. Alternatively, the Tn5 insertion could disrupt the expression of essential downstream cistrons in a polycistronic operon. Similar Southern blot analyses of genomic BamHI and EcoRI fragments indicate that none of the other virulence factor genes represented by the other Tn5-insertion mutants, are located within the 17 kb region defined by the first BamHI and the second EcoRI sites as shown in Figure 2a.

CONCLUSIONS

We have described the molecular cloning of pertussis toxin genes from virulent B. pertussis. The mapping of the subunit gene and a Tn5 DNA insertion affecting phenotypic expression of pertussis toxin at a distance of 1.3 kb from each other suggests that the genes probably are linked and may be expressed through a polycistronic operon, as has been shown in diphtheria toxin (14), E. coli heat labile toxin (15-18) and cholera toxin (16-20). Further characterization and DNA sequence data is needed to understand more about the expression, regulation of expression and the gene structure of pertussis toxin. The molecular cloning of the pertussis toxin gene opens the possibility of investigating some of these basic questions and developing a safer vaccine through genetic engineering.

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REFERENCES

1. Munoz, J. J., Arai, H., Bergman, R. K. and Sadowski, P. L. (1981) Infect. Immun. 33, 820-826.
2. Munoz, J. J. and Bergman, R. K. (1977) in Immunology Series (Rose, N., ed.) Vol. 4, pp. 1-235, Marcel Dekker, Inc., New York.
3. Munoz, J. J., Arai, H. and Cole, R. L. (1981) Infect.Immun. 32, 243-250.
4. Sato, H. and Sato Y. (1984) Infect. Immun. 46, 415-421.
5. Sato, Y., Kimura, M. and Fukumi, H. (1984) Lancet i, 122-126.
6. Manclark, C. R. and Cowell, J. L. (1984) in Bacterial Vaccines (Germanier, R., ed.) pp. 69-106, Academic Press, Inc., Orlando, Florida.
7. Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M. and Ishii, S. (1982) Biochemistry 21, 5516-5522.
8. Gill, D. M. (1978) in Bacterial Toxins and Cell Membranes (Jeljaszewics, J. and Wadstrom, T., eds.) pp. 291-332, Academic Press, New York.
9. Katada, T. and Ui, M. (1982) Proc. Natl. Acad. Sci. USA 79, 3129-3133.
10. Tamura, M., Nogimori, K., Yajima, M., Ase, K. and Ui, M. (1983) J. Biol. Chem. 258, 6756-6761.
11. Bokoch, G. M., Katada, T., Northup, J. K., Hewlett, E. L. and Gilman, A. G. (1983) J. Biol. Chem. 258, 2072-2075.
12. Hsia, J. A., Moss, J., Hewlett, E. L. and Vaughan, M. (1983) J. Biol. Chem. 259, 1086-1090.
13. Murayama, T. and Ui, M. (1983) J. Biol. Chem. 258, 3319-3326.
14. Kaczorek, M., Delpeyroux, F., Chenciner, N., Streek, R. E., Murphy, J. R., Boquet, P. and Tiollais, P. (1983) Science 221, 855-858.

15. Yamamoto, T. and Yokota, T. (1983) J. Bacteriol. 155, 728-733.
16. Yamamoto, T., Nakazawa, T., Miyata, T., Kaji, A. and Yokota, T. (1984) FEBS Lett. 169, 241-246.
17. Yamamoto, T., Suyama, A. Mori, N., Yokota, T. and Wada, A. (1985) FEBS Lett. 181, 377-380.
18. Pearson, G. D. N. and Mekalanos, J. J. (1982) Proc. Natl. Acad. Sci. USA 79, 2976-2980.
19. Mekalanos, J. J., Swartz, D. J., Pearson, G. D. N., Harford, N., Groyne, F. and de Wilde, M. (1983) Nature (London) 306, 551-557.
20. Lockman, H. and Kaper, J. B. (1983) J. Biol. Chem. 258, 13722-13726.
21. Weiss, A. A., Hewlett, E. L., Myers, G. A. and Falkow, S. (1983) Infect. Immun. 42, 33-41.
22. Munoz, J. J., Bernard, C. C. A. and Mackay, I. R. (1984) Cell. Immunol. 83, 92-100.
23. Howard, R. J., Raum, M. G., Maloy, W. L., Kao, V. and Coligan, J. E. (1984) Mol. Biochem. Parasitol. 12, 237-246.
24. Maxam, A. M. and Gilbert, W. (1980) Methods Enzymol. 65, 499-560.
25. Hull, R. A., Gill, R. E., Hsu, P., Minshew, B. H. and Falkow, S. (1981) Infect. Immun. 33, 933-938.
26. Casadaban, M. J., Chou, J. and Cohen, S. N. (1983) J. Bacteriol. 143, 971-980.
27. Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York.
28. Hanahan, D. (1983) J. Mol. Biol. 166, 557-580.
29. Woods, D. (1984) Focus 6,3, 1-3.
30. Kozak, M. (1983) Microbiol. Rev. 47, 1-45.
31. von Heijne, G. (1983) Eur. J. Biochem. 133, 17-21.
32. von Heijne, G., (1985) J. Mol. Biol. 184, 99-105.
33. Perlman, D. and Halvorson, H. O. (1983) J. Mol. Biol. 167, 391-409.
34. Shine, J. and Dalgarno, L. (1975) Nature (London) 254,34-38.
35. Pugsley, A. P. and Schwartz, M. (1985) FEMS Microbiol. Rev. 32, 3-38.
36. Michaelis, S. and Beckwith, J. (1982) Ann. Rev. Microbiol. 36, 435-465.
37. Weiss, A. A. and Falkow, S. (1984) Infect. Immun. 43, 263-269.
38. Weiss, A. A. and Falkow, S. (1983) J. Bacteriol. 153, 304-309.