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# Construction of a Nontoxic Fusion Peptide for Immunization against *Escherichia coli* Strains That Produce Heat-Labile and Heat-Stable Enterotoxins

JOHN D. CLEMENTS

*Department of Microbiology and Immunology, Tulane University School of Medicine, New Orleans, Louisiana 70112*

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**The 5′ terminus of the gene that codes for the heat-stable enterotoxin of *Escherichia coli* (ST) was genetically fused to the 3′ terminus of the gene that codes for the binding subunit of the heat-labile enterotoxin of *E. coli* (LT-B). The ST-encoding gene used for these studies was constructed synthetically with appropriate restriction sites to permit in-frame, downstream insertion of the oligomer. For this construction, maximum expression of ST antigenicity was obtained when a seven-amino-acid, proline-containing linker was included between the LT-B and ST moieties. The LT-B–ST fusion peptide was purified by affinity chromatography and consisted of a single polypeptide chain with an apparent molecular weight of 18,000 when examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. There was no evidence of multimer formation and no change in the mobility of the fusion peptide when it was boiled in SDS or in SDS with dithiothreitol. The LT-B–ST fusion peptide was nontoxic, and immunologic determinants of both LT and ST were recognized by antibodies to the native toxins. More importantly, the LT-B–ST fusion peptide was immunogenic. Animals immunized with crude or purified preparations containing the hybrid molecule produced antibodies that were able to recognize native toxin in vitro. Significantly, these antibodies were able to neutralize the biological activity of native ST.**

Enterotoxigenic *Escherichia coli* (ETEC) causes diarrheal disease by production of one or more enterotoxins. One of these is a high-molecular-weight heat-labile toxin (LT) which is immunologically and physicochemically related to cholera toxin (9). The other principal ETEC enterotoxin is a low-molecular-weight (ca. 2,000) peptide that is plasmid mediated and heat stable (ST) and functions by stimulating guanylate cyclase (2, 12, 14, 32). The relative contribution of each of these two enterotoxins of *E. coli* to the global problem of *E. coli*-mediated diarrheal disease has never been clearly established. What is known is that in some specific regions, ST-producing *E. coli* strains are the predominant ETEC strains identified in indigenous populations and in foreign visitors to those areas (19). Furthermore, it has been shown that other genera of bacteria can produce enterotoxins immunologically related to both LT and ST, including *Salmonella* sp., noncholera vibrios, *Yersinia* sp., *Klebsiella* sp., and *Enterobacter* sp. (13, 20, 26).

Antibacterial antibodies are an important component of experimental immunity in protection against cholera and the related enterotoxigenic enteropathies. The role of antitoxic immunity in protection against diarrhea caused by these organisms is less well defined. Cholera toxin (CT) and LT are both highly immunogenic, and the role of antitoxic immunity based upon antibodies to these toxins or to their binding subunits has been the subject of numerous studies (4, 16, 19, 20, 21, 25, 33). However, most isolates of ETEC produce ST, either alone or in combination with LT. It is not clear whether or not immunity to ST could significantly influence the occurrence of diarrheal disease due to organisms that produce this toxin. Effective immunization against ST would require development of a suitable toxoid capable of eliciting neutralizing antibodies. Such a potential immunogen would need to be delivered in an appropriate manner to elicit production of antitoxic antibodies on the mucosal surfaces of the proximal small bowel.

Because of its small size, ST tends to be poorly immunogenic. It can, however, assume immunogenicity when coupled to an appropriate carrier in a hapten-carrier configuration. Furthermore, it has been demonstrated that ST can be chemically coupled to either LT or its binding subunit (LT-B) (17). An alternative approach to the development of a suitable ST toxoid involves construction of genetic ST-LT and ST-CT fusions. Such molecules potentially possess a number of important advantages over chemically produced conjugates (15, 28–30). These include a precisely defined and homogeneous protein structure and the possibility of delivering the antigens via live oral vaccine.

The goal of the study reported here was to construct a gene for ST synthetically, with unique restriction sites appropriately placed to permit insertion of the ST DNA fragment in frame, downstream from the gene for LT-B on pJC217 (6, 8), a plasmid that codes for the B subunit of LT. This was done so as to produce a fusion polypeptide carrying antigenic determinants of both LT and ST but without toxicity. This plasmid has potential for use as one component of a multivalent oral vaccine against bacterial enteric disease based upon use of attenuated *Salmonella* mutants as carriers of heterologous antigens to the secretory immune system (3, 5, 6, 8, 10, 11, 24, 27). The LT-B–ST fusion peptide so derived could be delivered directly to the target cells in the lymphoid follicles for stimulation of a mucosal antitoxic response effective against both enterotoxins of *E. coli*.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *E. coli* JM83(pJC217) *ara* $\Delta$*lac-pro strA thi* $\phi$80d*lacZ* $\Delta$M15 is a K-12 derivative transformed with a 3.5-kilobase plasmid (pJC217) which

contains the gene for production of LT-B (6, 8). Plasmid pPX1604 is a derivative of plasmid pJC217 and was a gift of Robert Brey, Praxis Biologics, Rochester, N.Y. A brief description of the construction of this plasmid is included in Results. *E. coli* JM83(pJC710) is a derivative of JM83 containing a plasmid which codes for production of an immunogenic LT-B/ST fusion peptide (this study).

**Isolation of plasmid DNA.** Plasmid DNA was isolated as described by Maniatis et al. (23).

**Restriction endonuclease digestion.** Restriction endonucleases and reagents were obtained from Bethesda Research Laboratories, Inc. Reactions were performed as prescribed by the manufacturer.

**DNA and DNA sequencing.** Oligonucleotides were synthesized on a Biosearch Cyclone DNA Synthesizer by the phosphoramidite method. DNA sequence determinations were made by the dideoxy-chain termination method described by Sanger et al. (31).

**Electrophoresis.** Agarose gel electrophoresis was performed on 1% horizontal slab gels in 0.04 M Tris–0.2 M sodium acetate–0.002 M EDTA (pH 7.8). Bacteriophage λ DNA fragments generated by *Hin*dIII digestion were used as molecular weight standards. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the technique of Laemmli (18). Each well contained 25 μg of protein. Gels were stained with Coomassie brilliant blue (0.1%).

**Transformation.** Transformation was performed as previously described (6, 8). The plasmid that codes for production of LT-B was purified from *E. coli* JM83(pJC217) as previously described (6).

**Purification of the LT-B–ST fusion peptide.** The LT-B–ST fusion peptide was purified from *E. coli* JM83(pJC710) by affinity chromatography using affinity-purified monospecific anti-LT-B covalently coupled to Sepharose 4B (Sigma Chemical Co.). Organisms were cultured overnight at 37°C with vigorous aeration and agitation after inoculation with $10^6$ viable bacteria per ml. The bacteria were harvested by centrifugation at 4°C, and the cells were suspended in TEAN buffer (0.05 M Tris, 0.001 M EDTA, 0.003 M $NaN_3$, 0.2 M NaCl [pH 7.5]) (7) and lysed by French pressure cell. The crude lysate was clarified by centrifugation, dialyzed against TEAN buffer, and after additional centrifugation, applied directly to the affinity column equilibrated with TEAN buffer. The column was washed extensively with TEAN, and the LT-B–ST fusion peptide eluted with 0.2 M glycine hydrochloride (pH 2.5) containing 0.2 M NaCl. The affinity-purified LT-B–ST fusion peptide was concentrated by ultrafiltration on Amicon PM-10 membrane filters and stored in TEAN at 4°C before use.

**Protein determinations.** Protein determinations were made by the method of Lowry et al. (22).

**ELISA.** Reagents and antisera for the enzyme-linked immunosorbent assay (ELISA) were obtained from Sigma. Samples for ELISA were serially diluted in phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 (PBS-Tween).

For determination of LT-B or ST antigen in the fusion peptide and ganglioside-binding ability, microtiter plates were precoated with 1.5 μg of mixed gangliosides (type III) per well in carbonate-bicarbonate coating buffer (pH 9.6) and then with samples serially diluted in PBS-Tween. For determination of LT-B antigen, reactions were further developed with affinity-purified monospecific goat hyperimmune antiserum to LT-B, together with rabbit anti-goat immunoglobulin G conjugated to alkaline phosphatase. For determination of ST antigen in the fusion peptide, reactions were further developed with hyperimmune anti-ST serum raised in rabbits in conjunction with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. Other samples were assayed in plates not coated with gangliosides, in which case samples were diluted in coating buffer and applied directly to the microtiter wells. Subsequent steps were as described above. To compare the relative antigenicity (RA) of the fusion polypeptide compared with that of LT-B, a sample regression line for regression of the antigen on absorbance was calculated. A linear regression line was drawn for each sample, and the value (in nanograms) of the linear midpoint of absorbance was determined. Each sample was evaluated separately by taking five datum points along the linear portion of each dilution curve and determining the slope, *y* intercept, and amount of antigen required to produce an absorbance value equal to that of the linear midpoint. The RA value demonstrates the presence of immunologically reactive antigen. Differences result from variations in binding or alterations in immunologically reactive sites.

For determination of antibodies to LT-B, microtiter plates were precoated with 1.5 μg of mixed gangliosides (type III) per well and then with 1 μg of purified LT-B per well. For determination of antibodies to ST, microtiter plates were precoated with 1 μg of purified ST (a gift from Donald C. Robertson, University of Kansas, Lawrence) per well. Sera from mice immunized with the fusion peptide (see below) or control sera were serially diluted in PBS-Tween and added to the microtiter wells. The presence of anti-LT-B or anti-ST immunoglobulin G was determined by using rabbit antiserum to mouse immunoglobulin G conjugated to alkaline phosphatase.

**Immunization.** Female CD-1 mice (Charles River Breeding Laboratories, Inc.) were immunized intraperitoneally with 0.1 ml of crude cell lysate from *E. coli* JM83(pJC710) or 0.1 mg of purified LT-B–ST fusion peptide in 0.1 ml of sterile saline. The crude lysate was filter sterilized through a 0.22-μm-pore-size filter and injected three times at weekly intervals. Animals immunized with the purified LT-B–ST fusion were injected twice, with a single priming dose followed by a boost at 1 week. Animals were killed and sera were collected 1 week after the final boost.

**Bioassays.** The suckling mouse assay for ST was performed essentially as described by Giannella (12). Newborn CD-1 suckling mice (1 to 3 days old) were separated from their mothers immediately before use and randomly divided into groups. Each mouse was inoculated intragastrically with 0.1 ml of purified LT-B–ST fusion polypeptide containing 10 μl of 2% Evans blue dye per ml. There were three animals in each group. At 3 h postinoculation, the mice were killed, the abdomens were opened, and the entire intestines were removed with forceps. The intestine from each mouse was weighed, and the ratio of the gut weight to that of the remaining carcass (G/C) was calculated. The mean G/C ratio was then determined for each group. G/C ratios of ≥0.09 are considered positive.

Toxin neutralization in the suckling mouse assay was determined as follows. A selected dose of toxin (100 ng) was mixed with an equal volume of sera from mice immunized with either crude lysate from *E. coli* JM83(pJC710) or purified LT-B–ST fusion peptide. The final dilution of the sera was 1/50. Following preincubation for 1 h at room temperature, samples were administered to groups of suckling mice as described above. The minimum amount of ST necessary to produce a positive response in the suckling mouse assay is 1 ng.

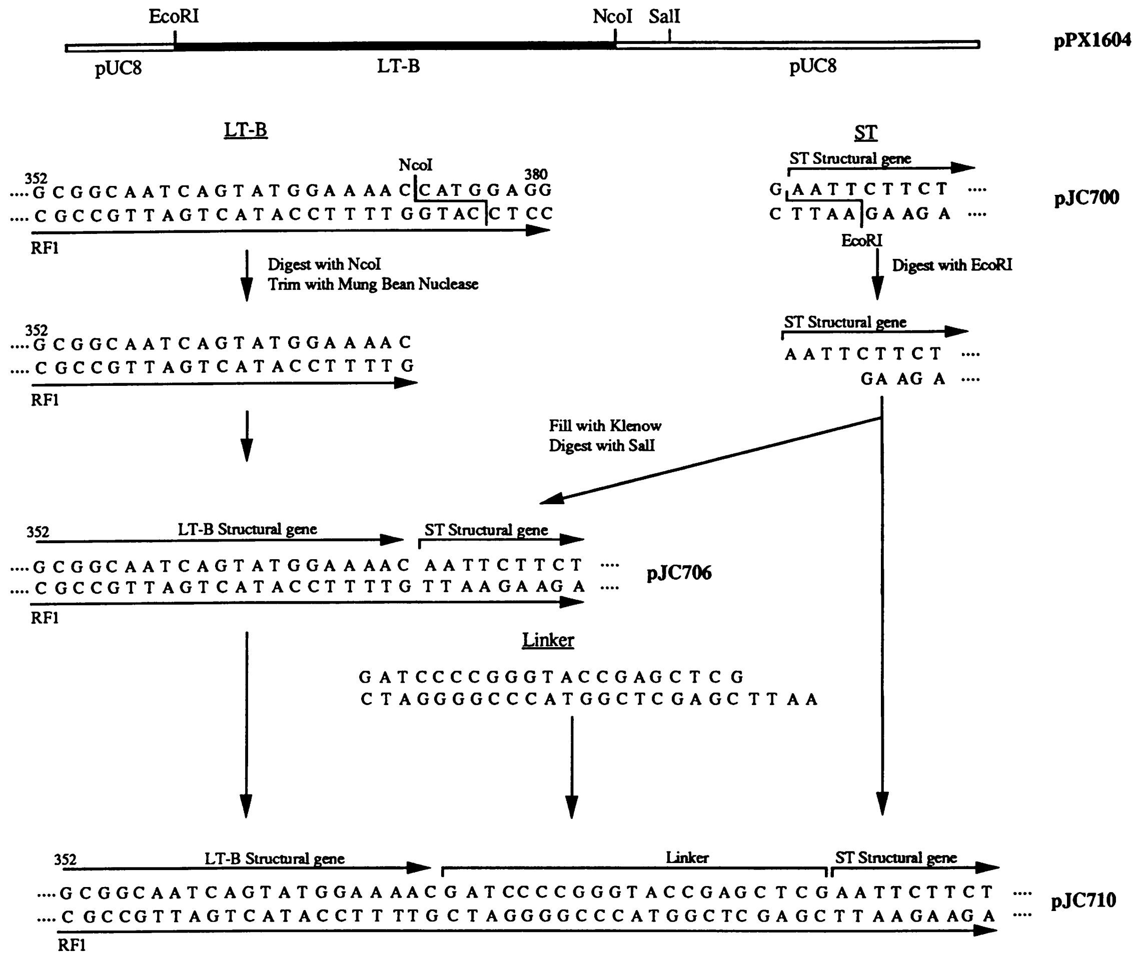


FIG. 1. Construction of the LT-B–ST gene fusion. A synthetic oligonucleotide constituting the structural gene for ST was cloned into plasmid pUC18 across the *Eco*RI and *Sal*I sites. This plasmid was designated pJC700 and was used as the source of the gene for ST. The gene for ST was purified from plasmid pJC700 and ligated into plasmid pPX1604. For the initial insertion, plasmid pJC700 was digested with *Eco*RI, filled with Klenow fragment to create a blunt end at the 5′ end of the gene for ST, and then digested with *Sal*I. Plasmid pPX1604 was digested with *Nco*I, and a blunt end was created with mung bean nuclease. This plasmid was subsequently digested with *Sal*I, thus permitting forced-cloning insertion of the gene for ST (plasmid pJC706). Two additional constructions were made with synthetic oligonucleotides that code for either a three-amino-acid (Ile-Pro-Gly) or a seven-amino-acid (Asp-Pro-Arg-Val-Pro-Ser-Ser) linker inserted between the gene for LT-B and the synthetic gene for ST. The construction using the seven-amino-acid linker is shown (plasmid pJC710). RF1, Reading frame 1.

**Guidelines used for recombinant DNA experiments.** The experiments reported here were performed under conditions specified in the *Guidelines for Recombinant DNA Technology* published by the National Institutes of Health, Bethesda, Md.

**Statistical analysis.** The standard error of the mean was calculated for all data, and means of variously immunized groups were compared by the Student $t$ test. Statistical significance was considered to be $P \leq 0.05$.

## RESULTS

**Construction of plasmid pJC710.** The plasmid that encodes LT-B was isolated from *E. coli* JM83(pJC217), a K-12 derivative transformed with a 3.5-kilobase plasmid (pJC217) which contains the gene for production of LT-B (6). This plasmid was modified to permit downstream, in-frame insertion of DNA sequences for expression of fusion peptides with LT-B at the carboxy terminus. First, plasmid DNA from pJC217 was purified by cesium chloride gradient centrifugation and digested with restriction endonuclease *Spe*I. This enzyme has a single recognition site within the LT-B-encoding gene, beginning at nucleotide 371, and recognizes the sequence A/CTAGT. The TAG sequence of this site constitutes the termination codon of the LT-B-encoding gene. This plasmid was then ethanol precipitated and suspended in TLE (0.01 M Tris, 0.001 M EDTA [pH 7.5]), and single-stranded ends were degraded with mung bean nuclease. A synthetic polylinker was inserted at that point with the sequence CCATGGAGGCCTTGATATCCTGAATGA CTGA. This modified LT-B plasmid (pPX1604) codes for intact, full-length LT-B with an additional nine amino acids at the carboxy terminus. The polylinker contains restriction sites for the enzymes *Nco*I, *Stu*I, and *Eco*RV and termination codons (TGA) in all three reading frames. Additional restriction sites downstream from the original polylinker are present in pJC217, including a *Sal*I site (Fig. 1).

The nucleotide sequence of the synthetic gene for ST used for this study was based upon the ST amino acid sequence determined by Aimoto et al. (1). It should be noted that the sequence used for ST is that of ST1a. That sequence is taken

as representative, since antisera to STla can completely neutralize the biological activities of both STla and STlb. We constructed a synthetic ST-encoding oligomer based upon that sequence, flanked by *Eco*RI and *Sal*I recognition sites. Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis and subsequent application to $C_{18}$ Sep-Pack cartridges. The complementary oligonucleotides were then annealed, purified, digested with *Eco*RI and *Sal*I, and further purified by electroelution from an agarose gel following electrophoresis. By using T4 DNA ligase, the synthetic gene for ST was then ligated into plasmid pUC18 across the *Eco*RI and *Sal*I sites. This plasmid was designated pJC700 and used as the source of the gene for ST for the remainder of these studies. The sequence of the ST-encoding oligonucleotide in this plasmid was confirmed by the dideoxy sequencing method (31).

The gene for ST was purified from plasmid pJC700 and inserted into the modified LT-B plasmid. For the initial insertion, plasmid pJC700 was digested with *Eco*RI, filled with Klenow fragment to create a blunt end at the 5′ end of the gene for ST, and then digested with *Sal*I (Fig. 1). The blunt-ended gene for ST was purified by electroelution following electrophoresis in agarose. The modified LT-B plasmid was digested with *Nco*I, and a blunt end was created with mung bean nuclease. This plasmid was subsequently digested with *Sal*I, thus permitting forced cloning insertion of the gene for ST. This plasmid was designated pJC706. *E. coli* JM83 was transformed with plasmid pJC706, and the sequence of the LT-B–ST junction was determined by the dideoxy sequencing method (31). The sequence of the LT-B–ST junction was as predicted. Transformants were then examined for the ability to synthesize ST-related antigen by ELISA. *E. coli* JM83 transformed with plasmid pJC706 did not synthesize an ST-related antigen detectable by ELISA (see Fig. 3).

It was not clear why an ST-related antigen was not detected although the DNA sequence was correct for production of an LT-B–ST fusion peptide. There were a number of possible explanations for this observation, the most likely of which was that presentation of the ST molecule in the fusion peptide was not appropriate for recognition by anti-ST serum in the ELISA. To address this problem, two additional constructions were made with synthetic oligonucleotides that code for either a three-amino-acid (Ile-Pro-Gly) or a seven-amino-acid (Asp-Pro-Arg-Val-Pro-Ser-Ser) linker inserted between the gene for LT-B and the synthetic gene for ST. The construction using the seven-amino-acid linker is shown in Fig. 1. The sequence of the LT-B–linker–ST junction was confirmed by the dideoxy sequencing method (31). The complete nucleotide sequence and the inferred amino acid sequence of the fusion peptide with the seven-amino-acid linker are shown in Fig. 2. *E. coli* JM83 was transformed with plasmids pJC708 and pJC710, which were derived from these constructions. Transformants were then examined for the ability to synthesize an ST-related antigen by ELISA. *E. coli* JM83 transformed with these plasmids synthesized an ST-related antigen detectable by ELISA (Fig. 3). Of the three constructs tested, the one with the seven-amino-acid linker between LT-B and ST was the most effective at presenting the ST antigen. The antigen produced by that clone was designated the LT-B–ST fusion peptide. Additional studies were conducted to characterize the immunologic and biologic properties of that material.

**Purification and characterization of the LT-B–ST fusion peptide.** Both LT and LT-B can be purified to homogeneity by direct application of clarified cell lysates from overnight cultures to gel filtration columns containing galactose as a constituent of the gel matrix (7, 9). The purified proteins can be subsequently eluted by application of 0.2 M galactose to the gel filtration column. This association is presumably a function of the ganglioside-binding properties of these two molecules. It was not clear whether or not the LT-B–ST fusion peptide could also be purified by this technique. The ST fusion could alter the configuration of the LT-B molecule

| ATG | AAT | AAA | GTA | AAA | TGT | TAT | GTT | TTA | TTT | ACG | GCG |
|---|---|---|---|---|---|---|---|---|---|---|---|
| met | asn | lys | val | lys | cys | tyr | val | leu | phe | thr | ala |
| TTA | CTA | TCC | TCT | CTA | TGT | GCA | TAC | GGA | GCT | CCC | CAG |
| leu | leu | ser | ser | leu | cys | ala | tyr | gly | ala | pro | gln |
| TCT | ATT | ACA | GAA | CTA | TGT | TCG | GAA | TAT | CGC | AAC | ACA |
| ser | ile | thr | glu | leu | cys | ser | glu | tyr | arg | asn | thr |
| CAA | ATA | TAT | ACG | ATA | AAT | GAC | AAG | ATA | CTA | TCA | TAT |
| gln | ile | tyr | thr | ile | asn | asp | lys | ile | leu | ser | tyr |
| ACG | GAA | TCG | ATG | GCA | GGC | AAA | AGA | GAA | ATG | GTT | ATC |
| thr | glu | ser | met | ala | gly | lys | arg | glu | met | val | ile |
| ATT | ACA | TTT | AAG | AGC | GGC | GCA | ACA | TTT | CAG | GTC | GAA |
| ile | thr | phe | lys | ser | gly | ala | thr | phe | gln | val | glu |
| GTC | CCG | GGC | AGT | CAA | CAT | ATA | GAC | TCC | CAA | AAA | AAA |
| val | pro | gly | ser | gln | his | ile | asp | ser | gln | lys | lys |
| GCC | ATT | GAA | AGG | ATG | AAG | GAC | ACA | TTA | AGA | ATC | ACA |
| ala | ile | glu | arg | met | lys | asp | thr | leu | arg | ile | thr |
| TAT | CTG | ACC | GAG | ACC | AAA | ATT | GAT | AAA | TTA | TGT | GTA |
| tyr | leu | thr | glu | thr | lys | ile | asp | lys | leu | cys | val |
| TGG | AAT | AAT | AAA | ACC | CCC | AAT | TCA | ATT | GCG | GCA | ATC |
| trp | asn | asn | lys | thr | pro | asn | ser | ile | ala | ala | ile |
| AGT | ATG | GAA | AAC | GAT | CCC | CGG | GTA | CCG | AGC | TCG | AAT |
| ser | met | glu | asn | asp | pro | arg | val | pro | ser | ser | asn |
| TCT | TCT | AAC | TAC | TGC | TGT | GAA | CTT | TGT | TGT | AAT | CCT |
| ser | ser | asn | tyr | cys | cys | glu | leu | cys | cys | asn | pro |
| GCC | TGT | ACA | GGA | TGT | TAC | GTA | TAG | | | | |
| ala | cys | thr | gly | cys | tyr | val | STOP | | | | |

FIG. 2. Nucleotide sequence of the fusion gene for LT-B–ST with the inferred amino acid sequence. The sequence shown is for the construction with a seven-amino-acid (Asp-Pro-Arg-Val-Pro-Ser-Ser) linker inserted between the gene for LT-B and the synthetic gene for ST. DNA sequence determinations were made by the dideoxy-chain termination method described by Sanger et al. (31).

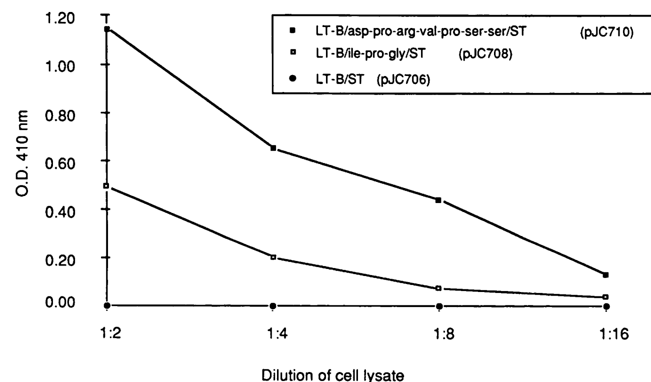


FIG. 3. ELISA comparison of ST antigenicity from three clones representing LT-B–ST fusions with no intervening linker (pJC706), a three-amino-acid (Ile-Pro-Gly) linker (pJC708), or a seven-amino-acid (Asp-Pro-Arg-Val-Pro-Ser-Ser) linker (pJC710) inserted between the gene for LT-B and the synthetic gene for ST. Comparisons were made on cell lysates from overnight cultures grown in ML medium (6) and lysed with lysozyme-EDTA and freeze-thawing. For additional details, see Materials and Methods. O.D., Optical density.

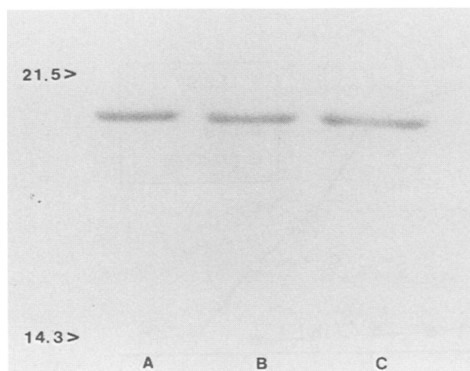


FIG. 4. Analytical discontinuous SDS-PAGE of the affinity-purified LT-B–ST fusion peptide. The purified LT-B–ST fusion peptide was shown to consist of a single polypeptide chain with an apparent molecular weight of 18,000. There was no evidence of multimer formation (A) as with LT-B and CT-B. There was no change in mobility of the fusion peptide when it was boiled in SDS (B) or SDS with dithiothreitol (C). SDS-PAGE was performed with 10% slab gels by the technique of Laemmli (18). Each well contained 25 μg of protein. The gels were stained with Coomassie brilliant blue (0.1%). The numbers to the left indicate molecular weights ($10^3$).

so as to prevent this interaction with the gel matrix. We had been able to detect the presence of the ST antigen in this peptide by ELISA by using plates precoated with mixed gangliosides (see above). One could make the assumption that a molecule that retains the ganglioside-binding activity of LT-B would also retain the galactose-binding capacity that permits agarose purification. This proved untrue with the fusion peptide studied here. Concentrated cell lysates were prepared (see Materials and Methods) and applied to gel filtration columns of Sepharose 4B. Since there is some lot-to-lot variation in the ability of agarose-containing gels to bind LT and LT-B, a lot was used which was previously established as suitable for purification of these proteins by this technique. After the crude lysate was applied, the column was washed and galactose was added to elute any material associated with the matrix as a function of galactose binding. In contrast to our experience with LT and LT-B purified by this technique, no LT-B or LT-B–ST fusion peptide was eluted from the column when galactose was applied, nor was there a reduction in the overall amount of LT-B or LT-B–ST antigen in the material that passed through the column when examined by ELISA (data not shown).

As an alternative to galactose affinity chromatography, the LT-B–ST fusion peptide was purified by antibody affinity chromatography. A column was prepared with affinity-purified monospecific anti-LT-B covalently coupled to Sepharose 4B. The crude lysate was applied to the affinity column equilibrated with TEAN buffer. The column was washed extensively with TEAN, and the LT-B–ST fusion peptide eluted with 0.2 M glycine hydrochloride (pH 2.5) containing 0.2 M NaCl. This purification was possible because the fusion peptide was bound to the antibody on the column via its remaining LT-B epitopes.

SDS-PAGE (Fig. 4) showed that the purified LT-B–ST fusion peptide consists of a single polypeptide chain with an apparent molecular weight of 18,000. There was no evidence of multimer formation (Fig. 4A) as with LT-B and CT-B. There was no change in the mobility of the fusion peptide when it was boiled in SDS (Fig. 4B) or when it was boiled in SDS with dithiothreitol (Fig. 4C).

The affinity-purified LT-B–ST fusion peptide was then analyzed by ELISA (Fig. 5) and compared with LT-B for the ability to bind to ganglioside and to react with antisera to either LT-B or ST. For this experiment, LT-B and LT-B–ST were either serially diluted in coating buffer and applied directly to microtiter wells or serially diluted in PBS-Tween and applied to wells precoated with 1.5 μg of mixed gangliosides. Samples were then reacted with affinity-purified monospecific goat hyperimmune antiserum to LT-B and, for the fusion peptide, hyperimmune anti-ST serum raised in rabbits. A curve for each sample with each datum point representing the mean of three independent measurements is shown in Fig. 5. For comparative purposes, an RA index was calculated for each sample. To obtain this value, a linear regression line was drawn for each sample and the value (in nanograms) of the linear midpoint of absorbance was determined (see Materials and Methods).

The LT-B–ST fusion peptide was first compared with LT-B for the ability to bind to ganglioside and to react with antiserum to LT-B. There was a 40-fold difference between the RA value obtained for LT-B when bound directly to the microtiter plate (RA, 36) and that obtained for LT-B when bound to gangliosides (RA, 0.86) (Fig. 5A). Moreover, there was no difference between the RA value obtained for the LT-B–ST fusion peptide when bound directly to the microtiter plate (RA, 167) and that obtained for the LT-B–ST fusion peptide when bound to gangliosides (RA, 172). This analysis also revealed a significant difference between the reactivity of the antiserum to LT-B with the fusion peptide when bound to ganglioside (RA, 172) and the reactivity of the antiserum to LT-B with LT-B when bound to ganglioside (RA, 0.86). These results demonstrate that incorporation of the ST moiety onto the carboxy terminus of LT-B significantly influenced the ganglioside-binding property of LT-B, an observation consistent with the observed lack of multimer formation determined by analysis in SDS-PAGE and with the apparent inability of the fusion peptide to bind to the agarose affinity matrix (see above). Also, a number of immunologic epitopes of LT-B were altered by the fusion. This is reflected in the different RA values obtained for LT-B (RA, 36) and the LT-B–ST fusion peptide (RA, 167) when they were bound directly to the microtiter plate.

The fusion peptide was then examined to determine the influence of ganglioside binding on the ability of the molecule to react with antiserum to ST. The ST moiety was less immunologically reactive with antiserum to ST when the fusion peptide was bound to gangliosides (RA, 354) than when it was bound directly to the microtiter plate (RA, 63) (Fig. 5B). This observation is consistent with a conformational shift in the LT-B–ST molecule upon binding to ganglioside. That conformational shift would presumably mask or alter ST epitopes of the fusion peptide and make them unavailable for reaction with anti-ST serum.

**Immunogenicity of the LT-B–ST fusion peptide.** The previous experiments showed that the LT-B–ST fusion peptide was antigenic; that is, the purified fusion peptide could react with antisera to either LT-B or ST. An important consideration for the use of this molecule as an immunoprophylactic was that it be capable of eliciting antibodies that were able to recognize and, more importantly, to neutralize the biological activity of native ST. Female CD-1 mice were immunized intraperitoneally with either 0.1 ml of a crude cell lysate from *E. coli* JM83(pJC710) or 0.1 mg of the purified LT-B–ST fusion peptide in 0.1 ml of sterile saline (see Materials and Methods). Sera from animals immunized with either the crude lysate or the purified fusion peptide were able to

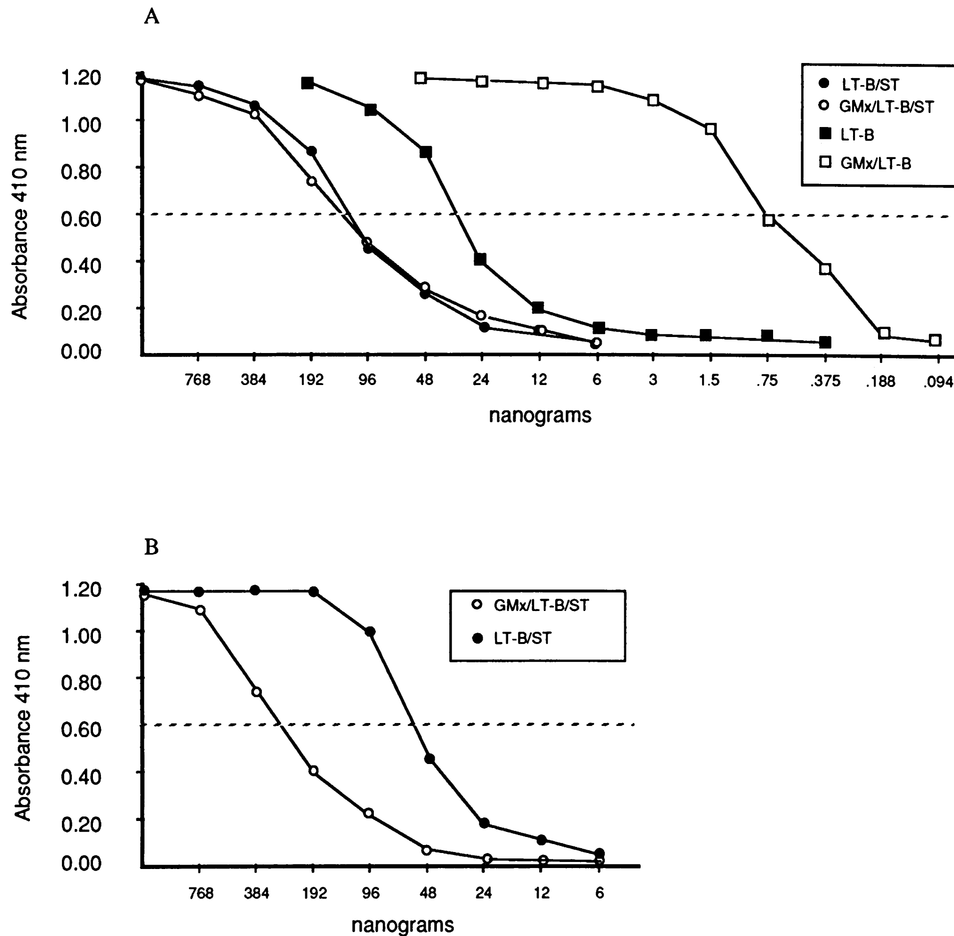


FIG. 5. ELISA evaluation of the LT-B–ST fusion peptide for the ability to bind to ganglioside and to react with antiserum to LT-B (A) and ST (B). For determination of LT-B or ST antigen in the fusion peptide and ganglioside-binding ability, microtiter plates were precoated with 1.5 μg of mixed gangliosides per well and then with samples serially diluted in PBS-Tween (open symbols). Other samples were assayed in plates not coated with gangliosides, in which case samples were diluted in coating buffer and applied directly to the microtiter wells (closed symbols).

recognize both LT-B and ST in the ELISA (Fig. 6). Reactivity to LT-B was greater; this probably reflects the highly immunogenic nature of LT-B.

An even more important consideration was the ability of antibodies to the fusion peptide to neutralize the biological activity of native ST. It was possible that alteration of the ST molecule sufficient to remove the toxicity would also render the molecule incapable of eliciting neutralizing antibodies. The neutralizing potential of antisera to the fusion peptide was evaluated in the suckling mouse assay. For the neutralization assay, 100 ng of ST was mixed with an equal volume of sera from mice immunized with either crude lysate from *E. coli* JM83(pJC710) or the purified LT-B–ST fusion peptide, and normal mouse serum was used as the control. Antisera to the fusion peptide, either purified or in crude cell lysates, was able to neutralize the biological activity of ST in the suckling mouse assay at the highest serum dilution tested (1:50). The G/C ratio was reduced from 0.100 with normal mouse serum to 0.081 with mouse anti-LT-B–ST crude lysate and 0.082 with mouse anti-LT-B–ST purified fusion peptide. G/C ratios of ≥0.09 were considered positive.

To be effective as an immunogen, and LT-B–ST fusion peptide must be not only immunogenic but also free of toxicity. Although LT-B derived from plasmid pJC217 has been shown to be free of residual toxicity (6), there was potential toxicity associated with the ST component of the fusion peptide. The purified LT-B–ST fusion peptide was analyzed in the suckling mouse assay and found to be free of ST-associated toxicity at levels of up to 10 μg, the greatest amount tested. The molecular weight of a monomer of the fusion peptide is approximately 18,000. Since the molecular weight of ST is approximately 2,000, the amount of ST in the fusion protein which was tested in the assay was 1.1 μg. That amount of ST is >1,000 times greater than the minimum effective dose of ST (1 ng).

## DISCUSSION

It has been shown that antitoxin is an important component of immunity to enterotoxigenic organisms such as *Vibrio cholerae* and ETEC. Evaluation of the role of antitoxic immunity based on immunization with toxoids derived from cholera toxin or *E. coli* LT is fairly straightforward. These are relatively high-molecular-weight, multisubunit proteins, and both are highly immunogenic. *E. coli* ST is also an important virulence determinant in enterotoxin-mediated diseases caused by these organisms. However, the small size and poor immunogenicity of the molecule have hampered

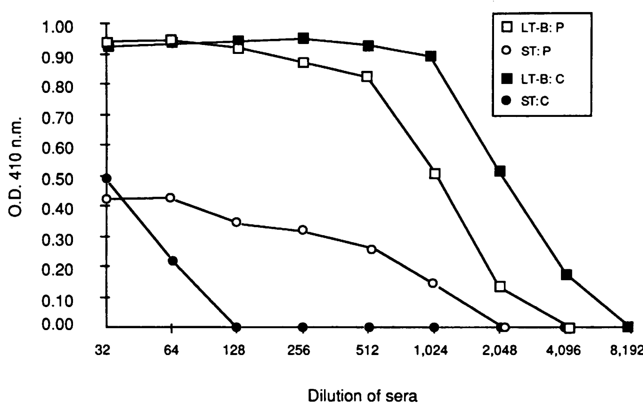


FIG. 6. ELISA evaluation of sera from animals immunized with either the crude lysate (C; closed symbols) or the purified fusion peptide (P; open symbols) for the ability to recognize both LT-B (squares) and ST (circles). See Materials and Methods for details. O.D., Optical density.

any serious study of whether or not immunization against ST could significantly influence the occurrence of diarrheal disease due to organisms that produce this toxin.

There have been a number of distinct approaches to the resolution of this problem. Like other low-molecular-weight peptides, ST can become immunogenic when coupled to an appropriate carrier in a standard hapten-carrier configuration. One attractive hypothesis was to use LT or LT-B as a carrier for ST in the development of a multivalent toxoid for protection against these two enterotoxins (17). This approach had the potential advantage of producing a molecule with immunologic determinants of both LT and ST and with the toxicity of the ST molecule abated as a function of the cross-linking. The disadvantages of this approach included the degree of manipulation, high cost, and inability to define the nature and degree of cross-linking of the end product. Moreover, the toxicity of ST in these preparations was reduced but not completely eliminated. An alternative approach to the development of a suitable ST toxoid involves construction of genetic LT-ST and CT-ST fusions. Such molecules potentially possess a number of important advantages over chemically produced conjugates (15, 28–30). These include a precisely defined and homogeneous protein structure and the possibility to deliver the antigens via live oral vaccine. To be useful, such genetic fusions must also retain immunogenicity for LT or CT, impart immunogenicity to ST, and be nontoxic.

A number of genetic fusions between ST and CT-B, LT-A, or LT-B have been reported. In the first of these (28, 30), two different lengths of the gene that encodes ST were fused to the carboxy terminus of the gene that codes for LT-A. The chimeric proteins resulting from these fusions possessed epitopes related to both LT-A and ST and were able to associate with native LT-B to form oligomeric structures capable of binding $G_{M1}$. These oligomeric structures reacted with monoclonal antibodies to LT-A, LT-B, or ST. No determination of toxicity or indication of immunogenicity was reported for these molecules. Guzman-Verduzco and Kupersztoch (15) created a genetic fusion between the 3′ terminus of the gene for ST and the 5′ terminus of the structural gene for LT-B. The ST–LT-B fusion peptide was recognized by anti-LT-B antibodies in Western blots (immunoblots), and both ST and LT-B were removed by adsorption to an anti-LT-B affinity column. Reactivity with antibodies to the ST component of the hybrid was not demonstrated, and the hybrid protein produced by these fusions possessed ST toxicity in the suckling mouse assay. The residual toxicity undoubtedly restricts the use of this product in a vaccine designed for use in humans. Sanchez et al. (29) have also reported the genetic fusion of a gene that encodes an ST-related decapeptide to the amino terminus of CT-B. This decapeptide contained a single mutation which substituted alanine for a disulfide-linked cysteine, a mutation designed to detoxify the ST moiety. This hybrid protein was nontoxic, was secreted from *Vibrio cholerae*, and retained the ability to bind to $G_{M1}$. The molecule contained antigenic determinants of both CT-B and ST and was able to elicit production of antibodies which could recognize native ST in an ELISA. The investigators did not, however, demonstrate that antibodies to this molecule were able to neutralize the biologic activity of ST. This is an important consideration, since removal of the epitopes associated with toxicity may, in such a small molecule, influence the ability of the molecule to elicit neutralizing antibodies.

In contrast to efforts reported by others, we constructed a genetic fusion between the carboxy terminus of LT-B and the amino terminus of ST. The gene for ST used for these studies was constructed synthetically with appropriate restriction sites to permit in-frame, downstream insertion of the oligomer. For this construction, maximum expression of ST antigenicity was obtained when a seven-amino-acid, proline-containing linker was included between the LT-B and ST moieties. It is interesting that in the absence of such a linker, no ST antigen was detected. It is probable that the ST determinants were masked by folding of the LT-B molecule. We found that animals immunized with the direct fusion peptide, that is, without an intervening linker, were unable to elicit antibodies capable of interacting with native ST (data not shown). This is clearly an important finding for those wishing to make similar constructions at the carboxy terminus of the LT-B molecule with other peptides and may have significance for use with carrier proteins other than LT-B. These studies also showed that incorporation of ST into the carboxy terminus of LT-B had a significant influence on the ability of LT-B to interact with $G_{M1}$. This was not true when ST was fused to the amino terminus of CT-B, as reported by Sanchez et al. (29). In any event, alteration of the $G_{M1}$-binding properties of LT-B by incorporation of ST into the carboxy terminus of the molecule did not diminish its utility as an immunogen against LT. Antibodies to the fusion peptide were fully immunoreactive to LT-B.

The LT-B–ST fusion peptide was nontoxic, and immunologic determinants of both LT and ST were recognized by antibodies to the native toxins. More importantly, the LT-B–ST fusion peptide was immunogenic. Animals immunized with either crude or purified preparations containing the hybrid molecule produced antibodies that were able to recognize native toxin in vitro. Significantly, these antibodies were able to neutralize, to some extent, the biological activity of native ST.

The purpose of these studies was to construct an LT-B–ST fusion peptide suitable for inclusion as one component of a live, oral, multivalent vaccine for typhoid fever, cholera, and the cholera-related enteropathies. Such a vaccine would be base upon the use of attenuated mutants of *Salmonella typhi* as carriers of heterologous antigens to the gut-associated lymphoid tissues (3, 5, 6, 8, 10, 11, 24, 27). This mechanism of antigen delivery has been shown to be effective for induction of mucosal antibodies to a variety of cloned antigens. We are currently evaluating methods of increasing

the immunogenicity of the ST component of these toxoids and stabilizing the gene for expression of the LT-B–ST fusion peptide in vivo.

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## LITERATURE CITED

1. **Aimoto, S., T. Takao, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani.** 1982. Amino-acid sequence of a heat-stable enterotoxin produced by human enterotoxigenic *Escherichia coli*. Eur. J. Biochem. **129**:257–263.
2. **Alderete, J. F., and D. C. Robertson.** 1978. Purification and chemical characterization of the heat-stable enterotoxin produced by porcine strains of enterotoxigenic *Escherichia coli*. Infect. Immun. **19**:1021–1030.
3. **Brown, A., C. E. Hormaeche, R. Demarco de Hormaeche, M. Winther, G. Dougan, D. J. Maskell, and B. A. D. Stocker.** 1987. An attenuated *aroA Salmonella typhimurium* vaccine elicits humoral and cellular immunity to cloned β-galactosidase in mice. J. Infect. Dis. **155**:86–92.
4. **Clemens, J. D., D. A. Sack, J. R. Harris, J. Chakraborty, M. R. Khan, B. F. Staanton, B. A. Kay, M. U. Khan, M. Yanus, W. Atkinson, A.-M. Svennerholm, and J. Holmgren.** 1986. Field trial of oral cholera vaccines in Bangladesh. Lancet **ii**:124–127.
5. **Clements, J. D.** 1987. Use of attenuated mutants of *Salmonella* as carriers for delivery of heterologous antigens to the secretory immune system. Pathol. Immunopathol. Res. **6**:137–146.
6. **Clements, J. D., and S. El-Morshidy.** 1984. Construction of a potential live oral bivalent vaccine for typhoid fever and cholera-*Escherichia coli* related diarrheas. Infect. Immun. **46**:564–569.
7. **Clements, J. D., and R. A. Finkelstein.** 1979. Isolation and characterization of homogeneous heat-labile enterotoxins with high specific activity from *Escherichia coli* cultures. Infect. Immun. **24**:760–769.
8. **Clements, J. D., F. L. Lyon, K. L. Lowe, A. L. Farrand, and S. El-Morshidy.** 1986. Oral immunization of mice with attenuated *Salmonella enteritidis* containing a recombinant plasmid which codes for production of the B subunit of heat-labile *Escherichia coli* enterotoxin. Infect. Immun. **53**:685–692.
9. **Clements, J. D., R. J. Yancey, and R. A. Finkelstein.** 1980. Properties of homogeneous heat-labile enterotoxin from *Escherichia coli* cultures. Infect. Immun. **24**:91–97.
10. **Curtiss, R., III, R. Goldschmidt, S. Kelly, M. Lyons, S. Michalek, R. Pastian, and S. Stein.** 1987. Recombinant avirulent *Salmonella* for oral immunization to induce mucosal immunity to bacterial pathogens, p. 261–271. *In* H. Kohler and P. T. LoVerde (ed.), Vaccines: new concepts and developments. Proceedings of the 10th International Convocation on Immunology. Longman Scientific and Technical, Harlow, Essex, Great Britain.
11. **Formal, S. B., L. S. Baron, D. J. Kopecko, O. Washington, C. Powell, and C. A. Life.** 1981. Construction of a potential bivalent vaccine strain: introduction of *Shigella sonnei* form I antigen genes into the *galE Salmonella typhi* Ty21a typhoid vaccine strain. Infect. Immun. **34**:746–750.
12. **Giannella, R. A.** 1976. Suckling mouse model for detection of heat-stable *Escherichia coli* enterotoxin: characteristics of the model. Infect. Immun. **14**:95–99.
13. **Giannella, R. A.** 1981. Pathogenesis of acute bacterial diarrheal disorders. 1981. Annu. Rev. Med. **32**:341–357.
14. **Greenberg, R. N., and R. L. Guerrant.** 1981. *E. coli* heat-stable enterotoxin. Pharmacol. Ther. **13**:507–531.
15. **Guzman-Verduzco, L. M., and Y. M. Kupersztoch.** 1987. Fusion of *Escherichia coli* heat-stable enterotoxin and heat-labile enterotoxin B subunit. J. Bacteriol. **169**:5201–5208.
16. **Kaper, J. B., and M. M. Levine.** 1988. Progress towards a vaccine against enterotoxigenic *Escherichia coli*. Vaccine **6**:197–199.
17. **Klipstein, F. A., R. F. Engert, and J. D. Clements.** 1982. Development of a vaccine of cross-linked heat-stable and heat-labile enterotoxins that protects against *Escherichia coli* producing either enterotoxin. Infect. Immun. **37**:550–557.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) **227**:680–685.
19. **Levine, M. M.** 1984. *Escherichia coli* infections, p. 187–235. *In* R. Germanier (ed.), Bacterial vaccines. Academic Press, Inc., New York.
20. **Levine, M. M., J. B. Kaper, R. E. Black, and M. L. Clements.** 1983. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. Microbiol. Rev. **47**:510–550.
21. **Levine, M. M., J. B. Kaper, D. Herrington, G. Losonsky, C. O. Tacket, J. G. Morris, B. Tall, and S. Cryz.** 1988. Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR. Lancet **ii**:467–470.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Maskell, D., F. Y. Liew, K. Sweeney, G. Dougan, and C. E. Hormaeche.** 1986. Attenuated *Salmonella typhimurium* as live oral vaccines and carriers for delivering antigens to the secretory immune system, p. 213–217. *In* F. Brown, R. M. Channok, and R. A. Lerner (ed.), Vaccines 86: new approaches to immunization. Developing vaccines against parasitic, bacterial, and viral diseases. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Pierce, N. F., W. C. Cray, Jr., J. B. Kaper, and J. J. Mekalanos.** 1988. Determinants of immunogenicity and mechanisms of protection by virulent and mutant *Vibrio cholerae* O1 in rabbits. Infect. Immun. **56**:142–148.
26. **Robertson, D. C.** 1988. Pathogenesis and enterotoxins of diarrheagenic *Escherichia coli*, p. 241–263. *In* J. A. Roth (ed.), Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington, D.C.
27. **Sadoff, J. C., W. R. Ballou, L. S. Baron, W. R. Majarian, R. N. Brey, W. T. Hockmeyer, J. F. Young, S. J. Cryz, J. Ou, G. H. Lowell, and J. D. Chulay.** 1988. Oral *Salmonella typhimurium* vaccine expressing circumsporozoite protein protects against malaria. Science **240**:336–340.
28. **Sanchez, J., T. R. Hirst, and B. E. Uhlin.** 1988. Hybrid enterotoxin LTA::STa proteins and their protection from degradation by *in vivo* association with B-subunits of *Escherichia coli* heat-labile enterotoxin. Gene **64**:265–275.
29. **Sanchez, J., A.-M. Svennerholm, and J. Holmgren.** 1988. Genetic fusion of a non-toxic heat-stable enterotoxin-related decapeptide antigen to cholera toxin B-subunit. FEBS Lett. **241**: 110–114.
30. **Sanchez, J., B. E. Uhlin, T. Grundström, J. Holmgren, and T. R. Hirst.** 1986. Immunoactive chimeric St-LT enterotoxins of *Escherichia coli* generated by *in vitro* gene fusion. FEBS Lett. **208**:194–198.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
32. **Smith, H. W., and C. L. Gyles.** 1970. The relationship between two apparently different enterotoxins produced by enteropathogenic strains of *E. coli* of porcine origin. J. Med. Microbiol. **3**:387–401.
33. **Svennerholm, A.-M., M. Jertborn, L. Gothefors, A. M. Karim, D. A. Sack, and J. Holmgren.** 1984. Local and systemic antibody responses and immunological memory in humans after cholera disease and after immunization with a combined B subunit-whole cell vaccine. J. Infect. Dis. **149**:884–893.