Construction of Stable LamB-Shiga Toxin B Subunit Hybrids: Analysis of Expression in *Salmonella typhimurium aroA* Strains and Stimulation of B Subunit-Specific Mucosal and Serum Antibody Responses†

GUO-FU SU, HIMANSHU N. BRAHMBHATT,‡* JÜRGEN WEHLAND,
MANFRED ROHDE, AND KENNETH N. TIMMIS

*Department of Microbiology, GBF, National Research Centre for
Biotechnology, Braunschweig, Germany*

The complete Shiga toxin B subunit and two N-terminal segments of the B subunit have been inserted into a cell surface exposed loop of the LamB protein, and expression of the hybrid proteins from three different promoter systems, i.e., (i) an in vitro-inducible *tac* promoter that provides high-level expression, (ii) the iron-regulated aerobactin promoter presumably induced in vivo under the iron-limiting conditions of the intestinal mucosal environment, and (iii) a synthetic, modified β-lactamase promoter providing moderate level constitutive expression, has been analyzed in *Escherichia coli*, *Salmonella typhimurium*, and attenuated antigen carrier strains of *S. typhimurium* (*aroA* mutants). The hybrid vaccine strains were used to immunize mice by the oral and intraperitoneal routes. *S. typhimurium aroA* mutants apparently have a membrane export defect which prevents the transport of LamB and its derivatives across the cytoplasmic membrane. High-level expression of hybrid proteins through use of the *tac* promoter proved deleterious to the vaccine strains and prevented the production of viable cells at reasonable cell densities. The lower levels of gene expression observed with the β-lactamase and aerobactin promoters did not have this effect. Immunization of mice with *S. typhimurium aroA* strains carrying the hybrid genes expressed from these two promoters resulted in significant B subunit-specific mucosal and serum antibody responses. This suggests that such expression systems may be useful when incorporated into candidate antidysentery live oral vaccines for inducing protection against the effect of Shiga toxin in infections caused by *Shigella dysenteriae* 1 and other Shiga toxin- or Shiga-like toxin-producing pathogens.

Bacillary dysentery is an invasive disease of the colon in humans and higher primates and is transmitted via the fecal-oral route. The disease is associated with poor hygiene, overcrowding, and stress, and hence, the majority of cases occur in regions of developing countries that have suboptimal sanitation. However, outbreaks have also been associated with war zones and mental institutions in developed countries. The causative agents are various species of *Shigella* and enteroinvasive strains of *Escherichia coli*. The current pandemic of bacillary dysentery caused by *Shigella dysenteriae* serotype 1 is characterized by high fatality rates and high rates of severe sequelae, such as hemolytic-uremic syndrome.

Current strategies to develop effective vaccines against shigellosis caused by *S. dysenteriae* 1 strains aim to induce protective mucosal immune responses against surface antigens, in particular the lipopolysaccharide (LPS) somatic (O) antigen, of the bacterium and focus on either the construction of hybrid attenuated *Salmonella* strains expressing the O antigen of *S. dysenteriae* 1 (30, 31) or the construction of attenuated strains of *S. dysenteriae* 1 itself (13). *S. dysenteriae* 1 strains produce a potent toxin, Shiga toxin (for reviews, see references 5, 21, 33, 46, and 51), a bipartite molecule

consisting of a toxic A subunit ($M_r = 32,000$) and a receptor-binding B subunit ($M_r = 7,700$) which associate noncovalently with an apparent subunit stoichiometry of one A chain and five B chains (10, 35). The A subunit inhibits eucaryotic protein synthesis by acting as an N-glycosidase that cleaves an adenine residue at nucleotide position 4324 of the 28S rRNA of the 60S ribosomal subunit (11, 41). The B subunit binds to its cell surface receptor, a glycolipid, globotriosyl ceramide (Gb3), carrying a terminal Galα(1-4)Gal moiety (20, 25). Chromosomal *stxA* and *stxB* genes which encode the Shiga toxin A and B subunits, respectively, have been mapped, cloned (45, 48), and sequenced (48). The role of Shiga toxin in pathogenesis has recently been explored by Fontaine and colleagues (12), who demonstrated that it influences the severity of bacillary dysentery by inducing colonic ischemia and inflation of the polymorphonuclear intestinal compartment during the infection process. The toxin can also damage the vascular endothelium of kidneys, and it has been suggested that it is the cause of the severe complications such as hemorrhagic colitis, hemolytic-uremic syndrome, and thrombotic thrombocytopenic purpura (22, 37). It is therefore desirable that antidysentery vaccines also induce immune protection against the Shiga toxin.

LamB is an *E. coli* outer membrane protein involved in the transport of maltose and maltodextrins into the cell (50). It also serves as a surface receptor for several bacteriophages, including bacteriophage lambda (7). Extensive genetic and protein structure characterization of LamB has led to the

---

* Corresponding author.
† This paper is dedicated to the memory of J.-P. Lecocq, acclaimed scientist and friend, who is sorely missed.
‡ Present address: CSIRO McMaster Laboratory, Private Bag No. 1, P.O. Glebe, Sydney, New South Wales 2037, Australia.

TABLE 1. Description of plasmids constructed in this study

Plasmid	Description
pSU108	*stxB* gene under the transcriptional and translational control of the thermoinducible $\lambda p_L/p_R$ promoter
pSU109	*stxB* gene subcloned into the *Bam*HI-*Sal*I sites of plasmid pGC1
pSU110	Same as pSU109, except that *Bam*HI and *Bgl*II sites have been created at the start and end of the structural *stxB* gene, respectively
pSU111	Same as pSU110, except that the *Bgl*II site has been created downstream of the 27th amino acid codon; *Bam*HI-*Bgl*II segment designated *stx27B*
pSU112	*stxB* gene inserted into the *Bam*HI site of *lamB* in plasmid pAJC264; the *lamB-stxB* fusion is under the control of the IPTG-inducible *tac* promoter
pSU113	Same as pSU112, except that the insert in the *Bam*HI site is *stx27B*
pSU114	Same as pSU112, except that the insert in *Bam*HI site is *stx17B* (encoding amino acids 11 to 27 of the *stxB* structural gene)
pSU115	Carries the *Bgl*II-*Cla*I *lamB* fragment fused to the *Cla*I site of *lacZ*; this fusion is under the control of the modified, synthetic β-lactamase promoter
pSU116	Carries *lamB* under the control of the modified, synthetic β-lactamase promoter
pSU117	Same as pSU116, except that *lamB* is replaced with *lamB-stxB* from pSU112
pSU118	Same as pSU116, except that *lamB* is replaced with *lamB-stx27B* from pSU113
pSU119	Same as pSU116, except that *lamB* is replaced with *lamB-stx17B* from pSU114
pSU120	Carries the *Bgl*II-*Cla*I *lamB* fragment fused to the *Cla*I site of *lacZ*; this fusion is under the control of the aerobactin promoter
pSU121	Carries *lamB* under the control of the aerobactin promoter
pSU122	Same as pSU121, except that *lamB* is replaced with *lamB-stxB* from pSU112
pSU123	Same as pSU121, except that *lamB* is replaced with *lamB-stx27B* from pSU113
pSU124	Same as pSU121, except that *lamB* is replaced with *lamB-stx17B* from pSU114
pSU207	Carries the *stxB-lacZ* gene fusion under the control of the aerobactin promoter
pSU208	Carries the *lacZ* gene under the control of the modified, synthetic β-lactamase promoter

identification of a unique site between amino acids 153 and 154, a region in the cell surface exposed loop of the protein, where insertion of peptide sequences can lead to their exposure on the bacterial cell surface (3, 6, 9). As a result, the LamB protein has been proposed as a carrier protein for the delivery of peptide epitopes by live bacteria to the immune system. The experiments reported here had as their goal the construction of *Salmonella typhimurium aroA* strains expressing LamB-B subunit hybrid proteins, which, when used as live immunogens, would elicit specific B subunit immune responses and antibodies directed against the epithelial cell receptor binding site of the B subunit and thereby block the endocytosis and hence the biological activity of the Shiga toxin.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study were as follows. *E. coli* pop6510 (*thr leu tonB thi lacY1 recA dex-5 metA supE*) and plasmid pAJC264, which carries the *lamB* gene under the control of the *tac* promoter and is inducible with isopropyl-β-D-thiogalactopyranoside (IPTG) (3), were a kind gift of M. Hofnung, Institute Pasteur, Paris, France. *S. typhimurium aro* mutants SL3235 (*aroA crr-401*), SL3261 (17), and the restriction-negative SL5283 were kindly provided by B. A. D. Stocker, Stanford University School of Medicine. Plasmid pDB74, which carries a mutated Shiga toxin locus with a Tn-mini-Kan transposon insertion in the *stxA* gene (4), was obtained from our laboratory. *E. coli* JM83 (52) [*ara* Δ(*lac-proAB*) *rpsL* φ80 *lac*ΔZM15], phagemid pGC1 (32), plasmid pcon1 (26), which carries the *lacZ* gene under the control of the aerobactin promoter, and *E. coli* DH5α [F⁻ φ80d*lac*ZΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoR* Δ(*lacZYA-argF*)] were kindly provided by Victor de Lorenzo. Plasmid pJLA503, carrying the thermoinducible cI857 gene (43), was a gift from J. E. G. McCarthy, and *E. coli aroA* AB2829 (34) was kindly provided by G. Dougan.

The plasmids constructed in this study are described in detail in Results and are also summarized in Table 1. Purified LamB protein and polyclonal LamB antiserum were kind gifts from M. Hofnung. Luria broth and Luria agar (29) were used as complete media for the routine growth of all strains, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-IPTG plates were prepared as described by Miller (29). When appropriate, bacterial growth media were supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml). Restriction endonucleases, T4 DNA ligase, DNA polymerase (Klenow enzyme), and all other enzymes were purchased either from Boehringer GmbH (Mannheim, Germany) or from New England Biolabs, Inc., Beverly, Mass., and were used in accordance with the recommendations of the manufacturer. Chemicals and salts were purchased from Sigma Chemical Co. (St. Louis, Mo.).

DNA manipulations. Preparation and handling of DNA was according to standard protocols (28). Bacterial transformation was performed as described by Hanahan (14), and DNA sequencing was done by the dideoxynucleotide chain termination method (40). Oligonucleotides were synthesized with an Applied Biosystems model 380B DNA synthesizer and purified on OPC columns (Applied Biosystems Inc.). Oligonucleotide-directed in vitro mutagenesis was performed as described previously (24), with the Bio-Rad Mutagene Phagemid in vitro mutagenesis kit. Polymerase chain reaction (PCR) was carried out by the method described by Saiki and colleagues (39).

SDS-PAGE. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method described by Schägger and Jagow (42). SDS-PAGE prestained molecular mass markers were either from Bio-Rad (97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa) or from Sigma (84.0, 58.0, 48.5, 36.5, and 26.6 kDa), as indicated in the figure legends.

Purification of B subunit protein. Overnight bacterial cultures grown at 30°C were diluted to an optical density at 560 nm (OD_{560}) of 0.4 and incubated further until the OD_{560}

reached 0.8. The culture was heat induced at 42°C for 4 h, and the cells were harvested by centrifugation (7,000 rpm for 15 min). The pellet was washed twice with 10 mM Tris-HCl (pH 8.0), resuspended in a solution containing 25% sucrose (wt/vol), 1 mM Na_2 EDTA, and 10 mM Tris-HCl (pH 8.0), and gently shaken for 10 min at 30°C. The cells were harvested and rapidly resuspended in ice-cold distilled water (osmotic shock treatment) and shaken gently for 10 min at 4°C. After centrifugation, the supernatant fraction (periplasmic fraction) was collected and the pellet was resuspended in 10 mM Tris-HCl (pH 8.0) (cytoplasmic fraction). Gel-loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) was added to both fractions, and the samples were boiled for 5 min prior to SDS-PAGE. Protein concentrations were determined by the method of Lowry et al. (27) with bovine serum albumin as the standard.

Purification of B subunit to homogeneity was performed by a two-step procedure involving fast protein liquid chromatography (FPLC) anion exchange and Superose-12 gel filtration. A 2-ml volume of periplasmic fraction was dialyzed overnight against 20 mM Tris-HCl buffer (pH 7.5) and loaded on a MonoQ HR 16/10 column (Pharmacia, Freiburg, Germany) equilibrated with the same buffer. The column was run at 1 ml/min and eluted with a linear NaCl gradient. B subunit was eluted at a salt concentration of 280 mM. Further purification was achieved by chromatography of the eluted B subunit on an FPLC Superose-12 prep-grade HR 16/50 gel filtration column which was equilibrated with phosphate-buffered saline (PBS) and run at a flow rate of 0.8 ml/ml. The first peak gave a homogeneous preparation of B subunit, as determined by subsequent SDS-PAGE and Western blot (immunoblot) analysis.

Western blotting of extracts. Western blotting was carried out essentially as described by Boulain and colleagues (3). Briefly, the bacterial strains were grown at 37°C in Luria broth supplemented with ampicillin. At an OD_{600} of 0.7, IPTG was added to a final concentration of 10^{-3} M for plasmids carrying the *tac* promoter (plasmids carrying the p_L/p_R promoters were heat induced at 42°C) and the bacteria were cultivated for a further 45 min. The cells were collected by centrifugation and resuspended at a 20-fold concentration in water. Cellular extracts (20 μl) from each suspension were diluted to a final volume of 40 μl with loading buffer (60 mM Tris [pH 6.8], 10% glycerol, 2% SDS, 0.05% bromophenol blue, 1 μl of β-mercaptoethanol), heat denatured for 5 min at 100°C, and run on SDS-PAGE. The proteins were transferred to a nitrocellulose filter with a semidry electroblotter, and LamB-B subunit fusion proteins were identified either with the preabsorbed B subunit-specific polyclonal antiserum or with one of the two B subunit-specific monoclonal antibodies. The antigen-antibody complexes were revealed by reaction with iodinated protein A and then by autoradiography.

Antibody production. (i) Monoclonal antibodies. Four 6-week-old female BALB/c mice were immunized at 3-week intervals with the FPLC-purified B subunit (100 to 200 μg of protein per injection) with Freund's complete adjuvant for the first injection and incomplete adjuvant for the two subsequent injections. Sera were tested by enzyme-linked immunosorbent assay (ELISA) and Western blotting. The spleen cells from the mouse giving the strongest reaction were fused with the myeloma cell line X63Ag8 (23). Colony supernatants were screened by ELISA with 96-well microtiter plates that were coated with approximately 0.5 μg of purified B subunit per well. Positive supernatants were further tested by Western blotting, and the corresponding hybridomas were cloned twice by limiting dilution.

(ii) Polyclonal antibodies. Two rabbits were immunized with the FPLC-purified recombinant B subunit (200 to 400 μg of protein per injection) by standard immunization procedures. The sera obtained were purified by repeated absorption with *E. coli* K-12 bacteria, and anti-B subunit antibodies were isolated by affinity chromatography on B subunit coupled CNBr-activated Sepharose 4B. Specific antibodies were eluted with 0.2 M sodium acetate (pH 2.7).

Immunoelectron microscopy. (i) Postembedding labeling. Bacteria were fixed with 0.5% formaldehyde and 0.2% glutaraldehyde (final concentration) in nutrient broth for 1 h on ice. After three washes with PBS (0.1 M phosphate buffer, 0.9% NaCl [pH 6.9]) containing 10 mM glycine to block free-aldehyde groups, the cells were embedded by the method of progressively lowering the temperature (38) by using Lowicryl K4M resin with the following slight modifications: (i) the infiltration step with 1 part ethanol to 1 part K4M resin was performed overnight, (ii) the infiltration step with 1 part ethanol to 2 parts K4M resin was done for 12 h, and (iii) infiltration with pure K4M resin was for 2 days, with several changes of the resin mixture. After polymerization of the samples at −35°C, ultrathin sections were incubated with protein A-purified cross-adsorbed antibody (180 μg of immunoglobulin G [IgG] protein per ml) for 16 h at room temperature and washed with PBS, followed by incubation of the sections with protein A-gold complexes (gold particle size, 10 nm; concentration giving an A_{520} of 0.02) produced by established procedures (47). Sections were subsequently rinsed with PBS containing 0.01% Tween 20 and then with distilled water. After being air dried, they were poststained with 4% aqueous uranyl acetate, pH 4.5, for 5 min.

(ii) Whole-cell labeling. The cells were harvested by centrifugation and resuspended in 0.5 ml of PBS. They were then adsorbed onto freshly prepared collodium-covered nickel grids, rinsed with distilled water, and air dried. The grids were incubated on drops of the cross-adsorbed and protein A-purified antibody (90 μg of IgG protein per ml) for 30 min at room temperature, washed with a mild spray of PBS from a plastic bottle, and subsequently incubated with protein A-gold complexes (gold particle size, 10 nm; concentration giving an A_{520} of 0.01) for 10 min at room temperature. The grids were subsequently rinsed with PBS containing 0.01% Tween 20 and then with distilled water and air dried.

Electron microscopy. Samples were examined with a Zeiss electron microscope CEM 902 or 10 B at an acceleration voltage of 80 kV and at calibrated magnifications.

Indirect immunofluorescence microscopy. Overnight bacterial cultures were washed twice with PBS and resuspended in PBS to an approximate density of 10^9 cells per ml. The following procedure was performed at room temperature. Glass coverslips (diameter, 12 mm) were coated for 5 min with polylysine (molecular mass, 90 kDa; 1 mg/ml in distilled water), washed twice with PBS, and incubated with bacteria (50 μl of bacterial suspension per coverslip) for 15 min in a wet chamber. After two washes with PBS, the coverslips were incubated for 1 h with 10 μl of either the polyclonal B subunit antiserum (1:1,000 diluted in PBS–10% fetal calf serum) or the monoclonal B subunit antibody (undiluted hybridoma supernatant). The coverslips were washed three times with PBS and incubated for 1 h with 10 μl of affinity-purified fluorescein-conjugated sheep anti-rabbit or rabbit anti-mouse IgG (1:50 diluted in PBS–10% fetal calf serum), respectively. After three washes with PBS, the coverslips

were imbedded with Moviol and analyzed by immunofluo-rescence microscopy with a Zeiss microscope.

Immunization and collection of sera and intestinal washings. Oral immunization was carried out essentially as described by Clements and colleagues (9). Briefly, for primary immunization with *S. typhimurium aroA* strains expressing various LamB-B subunit proteins, 8- to 10-week-old female BALB/c mice were immunized (four mice per immunization were fed orally two doses containing 10^{10} CFU of each strain on days 0 and 4 with the aid of a feeding tube). At 21 days after primary immunization, a booster injection was given; the mice were sacrificed 1 week later. Intestinal fluid and serum were collected from orally immunized mice, whereas serum only was collected from intraperitoneally (i.p.) immunized mice. Inocula for immunization were prepared as follows. Strains carrying *tac* promoter-based plasmids were grown as overnight cultures in Luria broth supplemented with ampicillin. The cells were then grown further in fresh medium until an OD_{600} of 0.7 was reached. IPTG was added (final concentration of 1 mM) to induce the *tac* promoter, and the cells were grown for a further 45 min. The cultures were washed twice in sterile normal saline and resuspended in an appropriate volume of saline to obtain a final concentration of 10^{11} CFU/ml. A 0.1-ml cell suspension was used for oral immunization. Cells were further diluted to 10^6 CFU/ml, and 0.1 ml of the cell suspension was used for i.p. immunization. Strains carrying β-lactamase promoter-based plasmids were grown as described above until the OD_{600} reached 1.0. The cells were washed and resuspended, and the suspension was used for immunization as mentioned above. Strains carrying aerobactin promoter-based plasmids were also grown as described above until the OD_{600} reached 0.3. 2,2'-Bipyridyl was added (final concentration of 100 μM) for induction of aerobactin promoter, and the cells were grown further for 3 h. The cultures were washed, resuspended, and used for immunization as stated above.

ELISA. Samples for ELISA were serially diluted in PBS (pH 7.2). For anti-B subunit determinations, microtiter plates were precoated with 1 μg of purified B subunit per well. The amount of serum anti-B subunit IgG plus IgM was determined with peroxidase conjugated goat anti-mouse IgG plus IgM (purchased from Dianova), and the amount of mucosal B subunit IgA was determined with peroxidase-conjugated goat anti-mouse IgA (obtained from Southern Biotechnology Inc.). The results were read with the Bio-Rad microplate reader (model 3550).

RESULTS

Plasmid construction for high-level expression and purification of B subunit. The 1.2-kb *Ssp*I fragment from plasmid pDB74 (Fig. 1), which carries the complete B subunit gene (*stxB*) and some flanking DNA, was subcloned into the Klenow fragment-filled *Eco*RI site of plasmid pJLA503. The resulting plasmid, pSU108, carries the *stxB* gene under the control of the lambda p_L and p_R promoters (in the native *stxAB* operon, the *stxB* region is transcribed from a promoter upstream of *stxA*). Uninduced and induced (induction by heating cultures to 42°C) whole-cell extracts of strain DH5α/pSU108 were electrophoresed on SDS-PAGE; two molecular weight forms of the B subunit were expressed at a high level (Fig. 2A, lanes 3 and 4). It was presumed that the higher-molecular-weight polypeptide was the premature form of the B subunit, while the lower-molecular-weight form was the mature form of the protein. After osmotic shock treatment of the cells, a procedure which would be

expected to release the periplasmic contents, the pellet and supernatant samples were electrophoresed on SDS-PAGE. As expected, the pellet was enriched for the higher-molecular-weight protein (Fig. 2A, lane 5), indicating that it was cytoplasmically localized, while the lower-molecular-weight polypeptide was found in the supernatant (Fig. 2A, lane 6). FPLC purification of the supernatant fraction followed by SDS-PAGE analysis (Fig. 2A, lane 7) showed that the purified sample contained the mature B subunit protein.

B subunit-specific polyclonal and monoclonal antibodies. The purified B subunit was used to immunize rabbits and mice to raise B subunit-specific polyclonal and monoclonal antibodies, respectively. The polyclonal antiserum was further purified by repeated adsorption with whole cells of *E. coli* K-12 carrying the plasmid vector pJLA503. The purity of the antiserum was assessed by Western blotting (Fig. 2B, lanes 1 and 2). Two monoclonal antibodies, StxBMb1 and StxBMb2, were identified, purified, and shown by Western blot analysis to react specifically with the B subunit (Fig. 2C, lanes 1 and 2; data shown only for StxBMb1).

Construction of LamB-B subunit gene fusions. Three different regions of the mature B subunit sequence were selected to generate LamB fusions. (i) The complete B subunit (69 amino acids) was selected. Studies to assess size limitations of foreign polypeptides that can be stably inserted into LamB revealed that 70 amino acid inserts formed the upper limit; larger insertions were found to be unstable and toxic to the host cells (8). (ii) The N-terminal 27 amino acids were selected. Harari and colleagues (16) have previously shown that antibodies directed against synthetic peptides of the N-terminal 26-amino-acid region of the Shiga toxin B subunit neutralized the cytotoxicity, enterotoxicity, and neurotoxicity of Shiga toxin to various degrees. It has also been shown that synthetic peptides representing the C-terminal region elicit protective responses in mice (15), although this region was not analyzed in our study. (iii) The 17-amino-acid region containing residues 10 to 27 of the mature protein was selected. A hydrophobicity-hydrophilicity computer plot of the B subunit sequence by the method of Hopp and Woods (18, 19) showed that the first 10 amino acids constitute a hydrophobic region; it has been suggested that such polypeptides inserted into LamB often lead to toxicity since they may act as "stop transfer" sequences and lead to a block in the export process (8).

As an intermediate step prior to the generation of *lamB-stxB* gene fusions, it was necessary to create *Bam*HI and *Bgl*II sites at appropriate positions in *stxB* to permit excision of precise fragments of *stxB* and their insertion into the *Bam*HI site of *lamB* to generate in-frame fusions. The Muta-gene phagemid in vitro mutagenesis kit from Bio-Rad was used to create these sites. The *Bam*HI-*Sal*I fragment carrying the entire *stxB* gene from plasmid pSU108 (Fig. 1) was subcloned between the *Bam*HI-*Sal*I sites of phagemid pGC1 (Fig. 1). The resulting phagemid, pSU109, was then used for site-directed mutagenesis. A synthetic oligonucleotide designated StxB-N (Fig. 3), which carries a *Bam*HI site just before the first codon (Thr) of the structural part of the *stxB* gene, was made. A second primer, StxB-C (Fig. 3), carries a *Bgl*II site downstream of the penultimate codon (codon 68 = Phe) of *stxB*. The generation of this site eliminates the *stxB* stop codon (TGA) and also replaces the last codon of *stxB* (+69 = Arg) by a serine residue. Phagemid pSU109 was mutagenized with primers StxB-N and StxB-C to give phagemid pSU110 (Fig. 1), which carries the newly created *Bam*HI and *Bgl*II sites flanking the *stxB* structural gene. A third oligonucleotide, StxB-3 (Fig. 3),
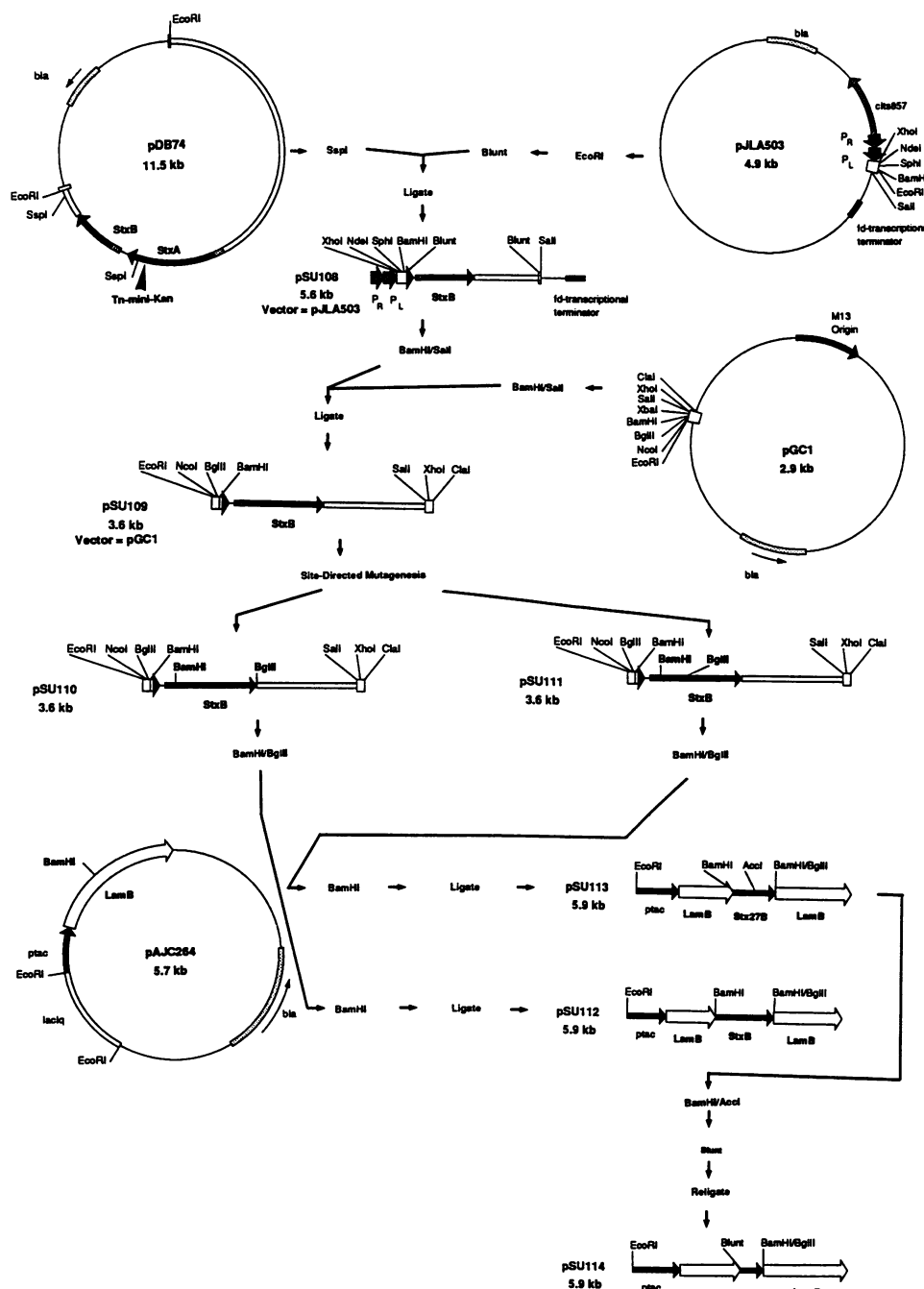
FIG. 1. Plasmid construction for high-level expression of B subunit and generation of LamB-B subunit fusions. For several plasmids, only the insert regions with relevant vector details are shown.

which carries a *Bgl*II site downstream of the 27th amino acid codon of *stxB* and replaces the 28th amino acid (Glu) by Asp, was synthesized. This primer was used in conjunction with primer StxB-N to mutagenize phagemid pSU109; the resulting phagemid, pSU111 (Fig. 1), carried the *Bam*HI site as described for phagemid pSU110 and a *Bgl*II site downstream of the 27th amino acid codon of *stxB*. All of the sites generated in phagemids pSU110 and pSU111 were confirmed by DNA sequencing. Phagemids pSU110 and pSU111 were cleaved with *Bam*HI-*Bgl*II, and the B subunit inserts were subcloned into the *Bam*HI site of plasmid pAJC264. Sub-

clones were screened by restriction digests to identify those in the correct orientation. The resulting plasmids pSU112 and pSU113 (Fig. 1) were found to carry the complete B subunit (designated *lamB-stxB*) and the 27-amino-acid region (designated *lamB-stx27B*), respectively. Nucleotide sequencing of the fusion junctions and the entire sequence between the restriction sites revealed that the sequences were as expected. The plasmid pSU113 was digested with *Bam*HI and *Acc*I, blunt ended with DNA polymerase (Klenow enzyme), and religated. This resulted in plasmid pSU114 (Fig. 1), which carries the 17-amino-acid region
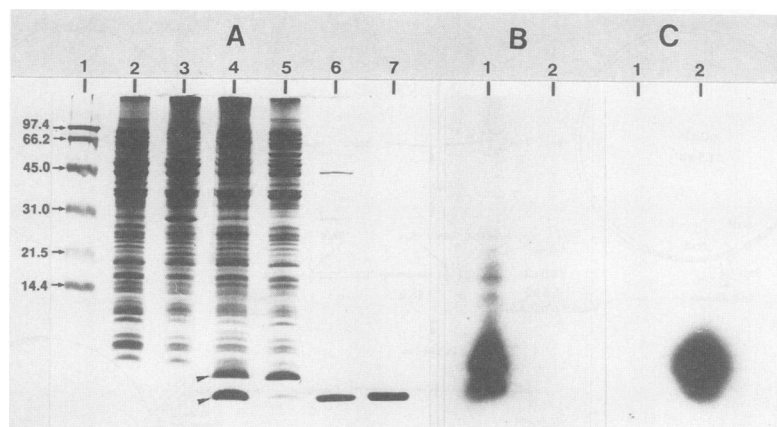
FIG. 2. (A) Coomassie blue-stained SDS-PAGE analyses of high-level expression and purification of B subunit. Lanes: 1, molecular weight markers; 2, heat-denatured bacterial extract from strain DH5α/pJLA503; 3 and 4, heat-denatured bacterial extracts from strain DH5α/pSU108 before and after induction with IPTG, respectively; 5 and 6, after osmotic shock treatment of strain DH5α/pSU108, the pellet and supernatant fractions, respectively; 7, FPLC-purified B subunit from the supernatant fraction shown in lane 6. The premature and mature forms of the B subunit are indicated by arrowheads. (B) Western blot showing reactivity of B subunit-specific polyclonal antiserum with B subunit. Heat-denatured extracts of IPTG-induced bacteria were electrophoresed on SDS-PAGE. Lanes: 1, strain DH5α/pSU108; 2, strain DH5α/pJLA503. (C) Western blot showing reactivity of StxBMb1 monoclonal antibody with B subunit. Heat-denatured extracts of IPTG-induced bacteria were electrophoresed on SDS-PAGE. Lanes: 1, strain DH5α/pJLA503; 2, strain DH5α/pSU108.

fused to LamB (designated *lamB-stx17B*). Each of these plasmids was transformed into the *E. coli* host strain pop6510 and the SL3235 *aroA* strain of *S. typhimurium*. Prior to transformation of SL3235, the plasmids were first passaged through the restriction-negative *S. typhimurium* SL5283.

Western blotting analyses of the expression of the LamB-B subunit hybrid proteins in *E. coli* K-12 and in *S. typhimurium aroA* SL3235. Whole-cell extracts were prepared with and without IPTG induction from *E. coli* pop6510 and *S. typhimurium* SL3235 carrying plasmids pSU112 (*lamB-stxB*), pSU113 (*lamB-stx27B*), pSU114 (*lamB-stx17B*), and pAJC264 (*lamB*) and electrophoresed on SDS-PAGE. Western blotting analysis of the gels using the preabsorbed B subunit polyclonal antiserum failed to reveal the LamB-StxB protein in uninduced cells (Fig. 4, lane 3) but did reveal the LamB-Stx27B and LamB-Stx17B proteins (Fig. 4, lanes 5

and 7, respectively). After induction, all three hybrid proteins were detected in large amounts (Fig. 4, lanes 4, 6, and 8, respectively), as were some protein degradation products, since the background in induced cells was higher than in uninduced or control cells. Results obtained with *E. coli* and *S. typhimurium* hosts were similar, and hence only the data for the *E. coli* host are shown.

Immunoelectron microscopy analysis of the expression of LamB-B subunit hybrid proteins in various *E. coli* and *S. typhimurium* strains. In order to assess whether the B subunit regions were expressed as LamB hybrids with the B subunit regions exposed on the bacterial cell surface, immunoelectron microscopy studies of whole cells and of thin bacterial

Oligonucleotide	DNA sequence
StxB-N	Pro Thr +2 +1 BamHI 5′ AATCAGGCGTCGGATCCGCACTTGCTGA 3′
StxB-C	Ser Phe BglII +69+68 5′ CTGAGCTATTCTGAGATCTCGAAAAATAAC 3′
StxB-3	lys Asp BglII +27+26 5′ TGTTGGTAAATAGATCTTTATCACCC 3′
LamB-8	BglII LamB --------► 5′ CTCAGGAGATCTAATGATGATTACTCTGCTC 3′
LamB-9	ClaI 5′ GCCAACATCGATGTTTTCCAG 3′

FIG. 3. Oligonucleotides synthesized and used for DNA sequencing, site-directed mutagenesis, or PCR.
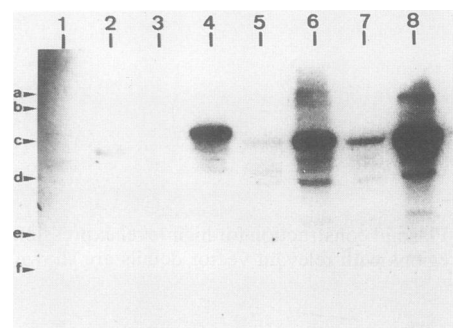

FIG. 4. Western blot showing expression of LamB-StxB, LamB-Stx27B, and LamB-Stx17B in *E. coli* pop6510. Heat-denatured bacterial extracts were electrophoresed on SDS-PAGE. The figure shows expression in *E. coli*. The lanes are in pairs showing expression before and after induction with IPTG. The proteins were revealed with the preabsorbed B subunit polyclonal antiserum. Lanes: 1 and 2, host strain/pAJC264; 3 and 4, host strain/pSU112 (LamB-StxB); 5 and 6, host strain/pSU113 (LamB-Stx27B); 7 and 8, host strain/pSU114. The molecular mass markers (a to f) are also shown, and they are 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa, respectively.

sections were carried out (Fig. 5A to C). The B subunit regions were revealed with preabsorbed polyclonal B subunit-specific antiserum. Thin-section studies showed that in *E. coli* K-12 strains expressing LamB-Stx17B and LamB-Stx27B, the hybrid protein was localized in the cytoplasmic compartment and in the bacterial membrane (Fig. 5A, g and h). The LamB-StxB protein, however, was seen predominantly as aggregates in the cytoplasmic compartment (Fig. 5A, f). In *S. typhimurium aroA* (SL3235) cells, no aggregates of the LamB-StxB protein were found and all of the fusion proteins were cytoplasmically localized (Fig. 5B, f, g, and h). Whole-cell analysis of the same strains showed that in *E. coli* K-12, the B subunit peptides of the fusion proteins, excepting that of LamB-StxB, were exposed on the bacterial cell surface (Fig. 5A, b, c, and d). In contrast, none of the B subunit regions of the hybrid proteins were exposed on the cell surface of *S. typhimurium aroA* (SL3235) cells (Fig. 5B, b, c, and d). These results raised the question of whether another feature of the *aroA* mutation or the *S. typhimurium* host was responsible for the observed membrane export defect in SL3235. In order to resolve these questions, the recombinant and control plasmids were transformed into *S. typhimurium* SL5283 (with no defect in *aro* genes), SL3261 (another *S. typhimurium aroA* mutant), and AB2829 (an *E. coli* K-12 *aroA* strain). The hybrid strains were analyzed by immunoelectron microscopy as described above. The results with SL5283 and AB2829 hosts were identical to those in *E. coli* K-12 (Fig. 5C; data for recombinant plasmids in AB2829 are not shown since they are identical to those in Fig. 5A), while the SL3261 host gave the same results as those observed for SL3235 (data not shown). These strains were also simultaneously analyzed with anti-LamB serum, and similar results were obtained (summarized in Table 2), i.e., in control and hybrid strains of *E. coli* K-12, *E. coli aroA*, and *S. typhimurium* SL5283, the LamB protein was detected on the bacterial cell surface, whereas neither of the *S. typhimurium aroA* mutants exported LamB to the outer membrane. These results demonstrate that LamB and LamB hybrids are expressed in *S. typhimurium* in a way similar to that found in *E. coli* K-12 (compare Fig. 5C and A) and that t he *aroA* defect is not responsible for the membrane export defect since the *E. coli aroA* strain also exported the LamB hybrids to the outer membrane. It is likely, therefore, that the *S. typhimurium aroA* strains SL3235 and SL3261 have additional mutations which cause the membrane export defect.

Indirect immunofluorescence analysis of expression of LamB-B subunit hybrid proteins in *E. coli* K-12 and in the *S. typhimurium aroA* strain. In order to assess whether the B subunit regions in LamB hybrids can be detected on the surfaces of whole cells by gentler treatment than that employed in electron microscopy studies, the *E. coli* K-12 and *S. typhimurium aroA* strains harboring the hybrid LamB-B subunit plasmids were analyzed by indirect immunofluorescence with the preabsorbed B subunit-specific polyclonal antiserum (Fig. 5D; data shown only for plasmids expressed in *E. coli* K-12). In phase-contrast microscopy of *E. coli* K-12, *E. coli aroA*, and *S. typhimurium* (SL5283) cells expressing the LamB-Stx17B and LamB-Stx27B proteins, the B subunit regions could be detected on the bacterial cell surface. Almost all of the bacterial cells observed were dense, suggesting that very few cells had lysed. Under phase-contrast microscopy, the intact bacteria appeared dense while the lysed cells appeared light (designated phase dense and phase light, respectively). However, cells harboring the LamB-StxB protein showed that a large number of

the bacteria had lysed and only these cells revealed the hybrid protein. This indicated that the LamB-StxB protein was localized in the cytoplasmic compartment and could be detected only when the cells had lysed. In the case of *S. typhimurium aroA* strains SL3235 and SL3261, none showed any fluorescence, except for phase-light cells. These results were thus in agreement with those obtained by immunoelectron microscopy.

Synthesis of a modified β-lactamase promoter and test for function. The *tac* promoter in plasmid pAJC264 gave rise to two major difficulties. (i) Upon induction with IPTG, the insert DNA was expressed at very high levels and the resulting protein product(s) proved toxic to the cells, preventing the cultivation of bacteria to greater than 10^8 live cells per ml of culture. For oral immunization of BALB/c mice, at least 10^8 live *S. typhimurium aroA* cells are required (8a). (ii) The *tac* promoter, which is inducible with IPTG, cannot be induced in vivo. We therefore chose to replace the *tac* promoter with (i) a synthetic, modified β-lactamase promoter which provides moderate-level, constitutive expression and (ii) the aerobactin promoter, which is induced under iron-limiting conditions such as those found in intestinal tissues and hence presumably works as an in vivo-inducible promoter. Figure 6A shows the DNA sequences of the β-lactamase promoter and a synthetic, modified version of it (Fig. 6B), which includes (i) an *Eco*RI site upstream of the −35 region and a *Bam*HI site following the ATG translation start codon, (ii) *Xho*I and *Nde*I sites flanking the ribosome binding site which permits variation of the translation initiation region sequence according to the required promoter strength, and (iii) removal of 10 bp from the translational initiation region in order to reduce the size of the oligonucleotides to be synthesized. The *Eco*RI-*Bam*HI fragment in plasmid pcon1 (Fig. 7) was replaced with the *Eco*RI-*Bam*HI fragment carrying the β-lactamase promoter to give plasmid pSU208. This plasmid was transformed into strain JM83, and the colonies were plated on X-Gal plates. All colonies obtained were blue, demonstrating that the synthetic β-lactamase promoter was functional and expressed β-galactosidase.

Replacement of the *tac* promoter in LamB and LamB-B subunit plasmids with β-lactamase and aerobactin promoters. A problem in replacing the *tac* promoter was that no convenient restriction enzyme sites exist between the *tac* promoter and the ATG translation start codon of *lamB* in plasmid pAJC264. A fragment encoding part of the signal sequence of *lamB* was therefore synthesized as a *Bgl*II-*Cla*I PCR fragment (Fig. 3; oligonucleotides LamB-8 and LamB-9 used for PCR) and was used to replace the *Bam*HI-*Cla*I fragment of plasmid pSU208 (Fig. 7). The resulting plasmid, pSU115, was cleaved with *Eco*RI-*Cla*I, and the β-lactamase promoter (including the PCR fragment) was used to replace the corresponding *Eco*RI-*Cla*I fragments in plasmids pAJC264 (*lamB*), pSU112 (*lamB-stxB*), pSU113 (*lamB-stx27B*), and pSU114 (*lamB-stx17B*), thereby generating plasmids pSU116, pSU117, pSU118, and pSU119, respectively (construction of pSU116 is shown in Fig. 7).

A similar approach was used to replace the *tac* promoter with the aerobactin promoter. The *Bam*HI-*Bgl*II *stxB* fragment from plasmid pSU110 (Fig. 7) was inserted into the *Bam*HI site of plasmid pcon1 to give plasmid pSU207, with the ATG initiating codon upstream of the *Bam*HI site. The *Bgl*II-*Cla*I PCR fragment was subcloned into the *Bam*HI-*Cla*I sites of plasmid pSU207 to give plasmid pSU120. The *Eco*RI-*Cla*I fragment from plasmid pSU120 was used to replace the corresponding fragments of plasmids pAJC264,
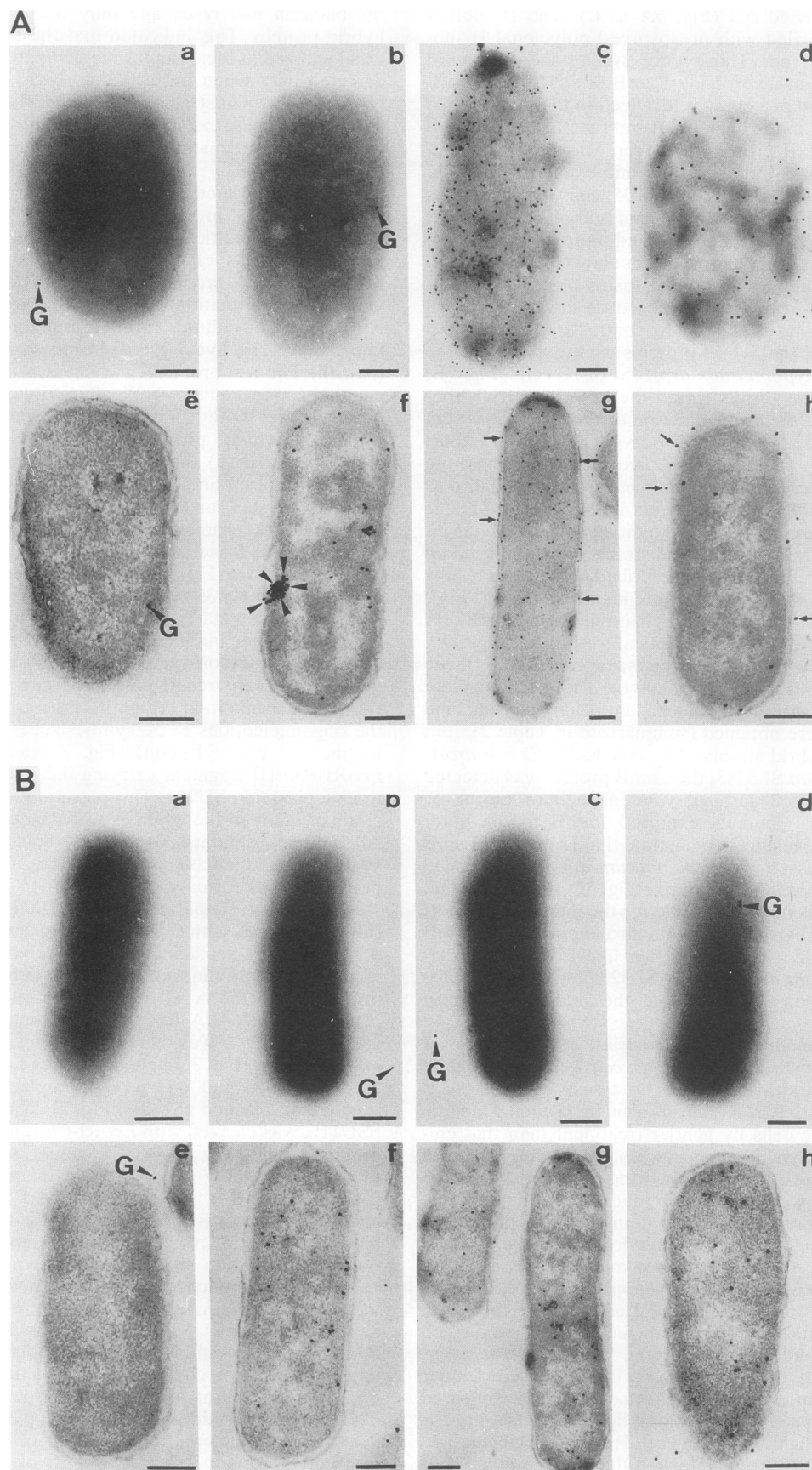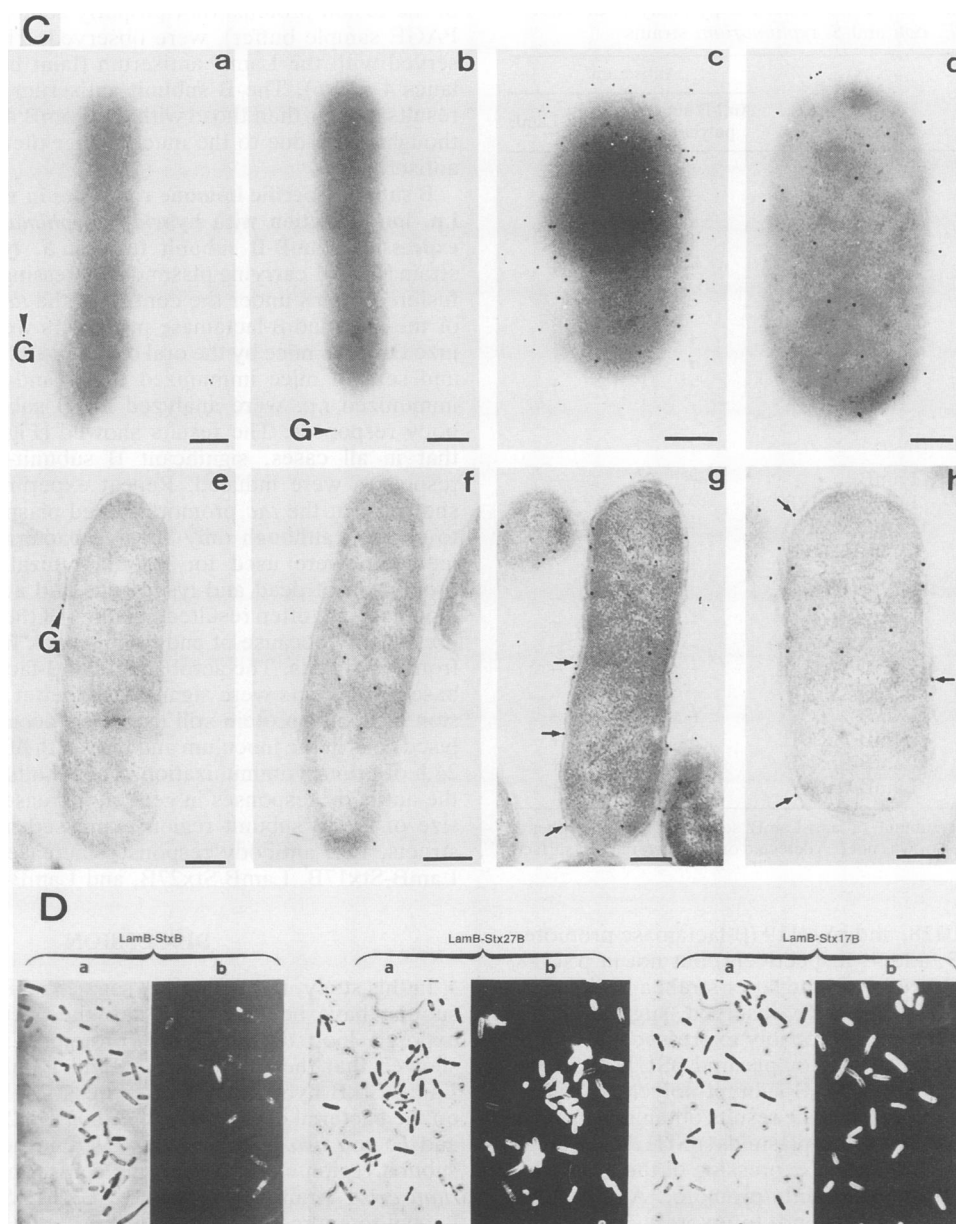
FIG. 5. Immunoelectron microscopy analysis of expression of the LamB-B subunit hybrid proteins in various bacterial hosts. The panels show whole cells (A through C, a to d) or thin bacterial sections (A through C, e to h) of *E. coli* K-12 strain pop6510 (A), *S. typhimurium* SL3235 (B), and *S. typhimurium* SL5283 (with no defects in *aro* genes) (C). The primary antibodies used in the labeling were gold-labeled anti-B subunit antibodies. Host strain/pAJC264 (a and e), host strain/pSU112 (LamB-StxB) (b and f), host strain/pSU113 (LamB-Stx27B) (c and g), and host strain/pSU114 (LamB-Stx17B) (d and h) are shown. (D) Indirect immunofluorescence analysis of *E. coli* K-12 (pop6510) cells expressing LamB-B subunit hybrid proteins. The same field is shown under phase contrast (a) and immunofluorescence (b).

pSU112, pSU113, and pSU114 to give plasmids pSU121, pSU122, pSU123, and pSU124, respectively (construction of pSU121 is shown in Fig. 7).

Western blotting analyses of expression of LamB-B subunit hybrids under the control of the modified β-lactamase and aerobactin promoters. *E. coli* K-12 and *S. typhimurium aroA* strains containing plasmid pSU207 were analyzed for expression of the B subunit–β-galactosidase fusion protein to determine whether the aerobactin promoter was functional (Fig. 7). Figure 8 shows the data for the expression of the B subunit–β-galactosidase fusion protein in both *S.*

typhimurium aroA strains (SL3235 and SL3261; data for *E. coli* K-12 not shown). In all strains, the fusion protein was expressed after induction with dipyridyl (Fig. 8, lanes 4 and 6), suggesting that the aerobactin promoter was functional. Some basal activity was also detected in uninduced cultures (Fig. 8, lane 5). The expression of LamB and LamB-B subunit hybrids in plasmids pSU116, pSU117, pSU118, and pSU119 (Fig. 7) was analyzed by Western blotting of whole-cell bacterial extracts with either the LamB or the B subunit polyclonal antisera. Figure 9A shows that the LamB and LamB-B subunit fusion proteins could be detected in plas-

TABLE 2. Summary of electron microscopy analysis of whole cells of *E. coli* and *S. typhimurium* strains

Strain	LamB-B subunit fusion	Antiserum[a]	
		Anti-B subunit polyclonal	Anti-LamB
E. coli			
K-12 pop6510	LamB	−	+
	LamB-Stx17B	+	+
	LamB-Stx27B	+	+
	LamB-StxB	−	+
aroA AB2826	LamB	−	+
	LamB-Stx17B	+	+
	LamB-Stx27B	+	+
	LamB-StxB	−	+
S. typhimurium			
aroA SL3235	LamB	−	−
	LamB-Stx17B	−	−
	LamB-Stx27B	−	−
	LamB-StxB	−	−
aroA SL3261	LamB	−	−
	LamB-Stx17B	−	−
	LamB-Stx27B	−	−
	LamB-StxB	−	−
SL5283	LamB	−	+
	LamB-Stx17B	+	+
	LamB-Stx27B	+	+
	LamB-StxB	−	+

[a] Gold-labeled anti-B subunit or anti-LamB serum was used. Either B subunit or LamB was (+) or was not (−) detected on the bacterial cell surface.

mids pSU116, pSU118, and pSU119 (β-lactamase promoter; Fig. 9A, lanes 2, 3, and 4, respectively) but not in pSU117 (Fig. 9A, lane 5). However, in the latter strain a background of smaller polypeptides could be observed, suggesting that the LamB-StxB protein was probably expressed but rapidly degraded, as observed earlier for plasmid pSU112. Similar results were obtained with the B subunit polyclonal antiserum (Fig. 9B). Figure 10 shows the results obtained in similar Western blotting analysis for plasmids pSU121, pSU122, pSU123, and pSU124, in which expression of the proteins is under the control of the aerobactin promoter. All plasmids, except for pSU122, were found to express LamB and LamB-B subunit fusion proteins after induction with dipyridyl. In Fig. 10B, some high-molecular-weight bands (lanes 4, 6, and 8), which were thought to be aggregated multimers

of the fusion proteins (not properly solubilized in the SDS-PAGE sample buffer), were observed. They were also observed with the LamB antiserum (faint bands in Fig. 10A, lanes 4 and 6). The B subunit antiserum consistently gave results clearer than those with the LamB antiserum and was thought to be due to the much higher titer of the B subunit antiserum.

B subunit-specific immune responses in mice after oral and i.p. immunization with hybrid *S. typhimurium* aroA strains expressing LamB-B subunit fusions. *S. typhimurium* aroA strain SL3261 carrying plasmids expressing LamB-B subunit fusion proteins under the control of the *tac*, the aerobactin, or the modified β-lactamase promoters were used to immunize BALB/c mice by the oral or i.p. route. Intestinal washes and sera of mice immunized orally and the sera of mice immunized i.p. were analyzed for B subunit-specific antibody responses. The results showed (Fig. 11A through C) that in all cases, significant B subunit-specific antibody responses were induced. Repeat experiments consistently showed that the *tac* promoter-based plasmids were difficult to control; although only 10^5 live bacterial cells per ml of inoculum were used for i.p. immunization, a significant proportion of dead and lysed cells had accumulated in the inoculum and often resulted in killing of the mice within 24 h, presumably because of endotoxin shock from LPS released from lysed cells. The aerobactin and β-lactamase promoter-based constructs were significantly better, although expression of hybrid protein still led to the accumulation of some lysed cells in the inoculum and the death of some mice within 24 h of primary immunization. The results also showed that the antibody responses in general increased with increasing size of the B subunit region expressed in the LamB constructs, i.e., antibody responses increased in the order of LamB-Stx17B, LamB/Stx27B, and LamB-StxB.

DISCUSSION

In this study, three different segments of the Shiga toxin B subunit have been inserted into the bacterial cell surface exposed loop of the LamB protein. Expression studies showed that the LamB-Stx17B and LamB-Stx27B but not LamB-StxB hybrid proteins express the B subunit regions on the bacterial cell surface of *E. coli* K-12, *S. typhimurium*, and *E. coli* aroA strains. However, in no case was the B subunit region expressed on the cell surface of *S. typhimurium* aroA strains SL3235 and SL3261. *S. typhimurium* is known to express its own LamB protein (36, 44), and it has been shown that monoclonal antibodies raised against LamB of *E. coli* are immunologically cross-reactive with LamB of *S. typhimurium* (2). However, in the electron microscopy
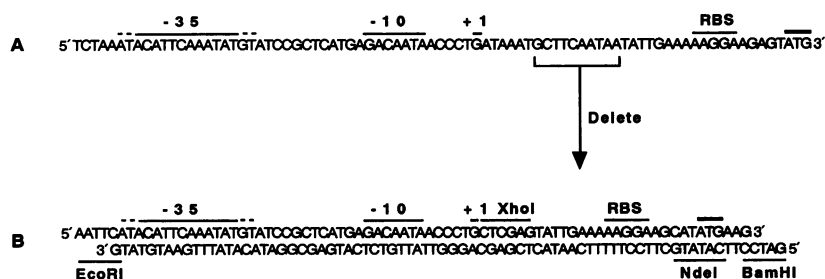
A 5′ TCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATG 3′

-35   -10   +1   RBS

Delete

B 5′ AATTCATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGCTCGAGTATTGAAAAAGGAAGCATATGAAG 3′
3′ GTATGTAAGTTTATACATAGGCGAGTACTCTGTTATTGGGACGAGCTCATAACTTTTTCCTTCGTATACTTCCTAG 5′

-35   -10   +1 XhoI   RBS

EcoRI   NdeI   BamHI

FIG. 6. In vitro synthesis of a synthetic modified β-lactamase promoter. Nucleotide sequences of the native β-lactamase promoter (A) and the synthetic, modified version (B). The −35 and −10 regions, the +1 transcriptional start site, the ribosome binding site (RBS), and the ATG translation start codon are shown. The newly introduced *Eco*RI, *Xho*I, *Nde*I, and *Bam*HI restriction enzyme sites are also indicated.
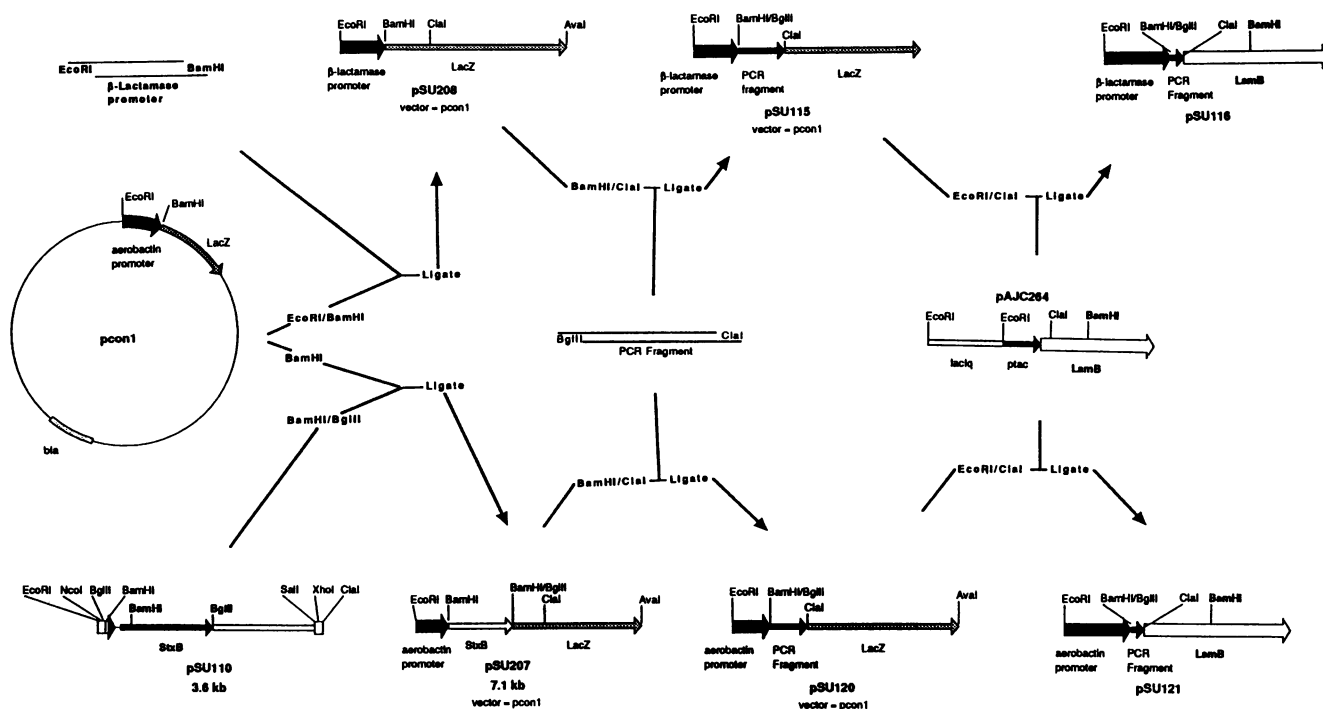
FIG. 7. Construction of plasmids for the replacement of the *tac* promoter with the synthetic modified β-lactamase or the aerobactin promoters.

and indirect immunofluorescence studies with LamB antiserum, only the *E. coli* K-12, *E. coli aroA*, and *S. typhimurium* SL5283 hosts exhibited LamB on the bacterial cell surface (Table 2), indicating that failure to surface express the LamB protein in the *aroA* mutants of *S. typhimurium* was not due to the heterologous nature of the *E. coli* LamB protein. The nature of the defect in *S. typhimurium aroA* strains SL3235 and SL3261 which leads to a block in the export of LamB and the hybrid proteins to the bacterial outer membrane is
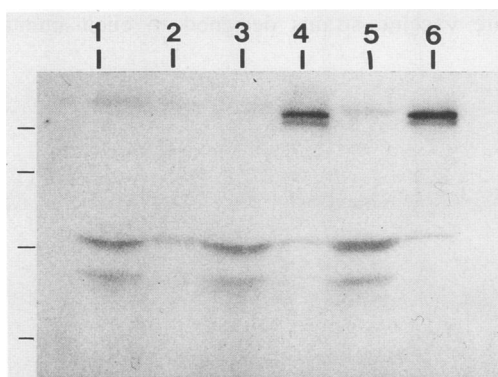

FIG. 8. Western blot showing expression of B subunit–β-galactosidase fusion protein under the control of the aerobactin promoter in plasmid pSU207 in *S. typhimurium aroA* strains SL3235 and SL3261. Heat-denatured bacterial extracts are electrophoresed, and the fusion proteins are revealed by B subunit-specific polyclonal antiserum. Lanes shown are in pairs before and after induction with dipyridyl. Lanes: 1 and 2, strain SL3261/pcon1 as a negative control; 3 and 4, strain SL3261/pSU207; 5 and 6, strain SL3235/pSU207. The molecular mass markers are indicated on the left, and from top to bottom they are 97.4, 66.2, 45.0, and 31.0 kDa.

not clear but it seems likely that the *S. typhimurium aroA* strains have additional mutations which cause the defect in the export process. These strains are not, however, defective in the transport of other outer membrane proteins normally found in wild-type *S. typhimurium*, and hence, it seems unlikely that any of the "chaperone" proteins of the Sec-mediated protein translocation pathway (for reviews on the Sec pathway, see reference 1) are mutated. Recent studies aimed at the dissection of the process of LamB export into membrane targeting and transmembrane translocation steps showed that apart from the proteins involved in the Sec pathway of protein export, another factor(s), such as a protease-sensitive membrane receptor, is involved (49). It is possible that one or more such factors have undergone mutations in these strains. It is to be expected that transfer of the *aroA* mutation to a clean *S. typhimurium* background will yield an attenuated vaccine strain that exports LamB fusion proteins to the cell surface.

The LamB-StxB fusion polypeptide proved to be toxic for *E. coli* and *S. typhimurium* cells and formed large intracytoplasmic aggregates. It is possible that either the large size of the inserted polypeptide (69 amino acids) or the strong hydrophobic domains in the C-terminal region of the B subunit protein affect the folding and/or assembly of the protein. Each hydrophobic polypeptide would, however, be expected to exhibit unique characteristic features, and not all such regions, if inserted into LamB, would necessarily perturb membrane folding and/or assembly. In fact, the first 10-amino-acid region of the StxB protein exhibits a strong hydrophobic domain, but it seems not to significantly affect LamB folding and assembly, since the LamB-Stx17B and LamB-Stx27B proteins were both stably expressed in both *E. coli* and *S. typhimurium* strains.

The *tac* promoter-based vector for the expression of LamB hybrids, developed by Hofnung and colleagues, ap-
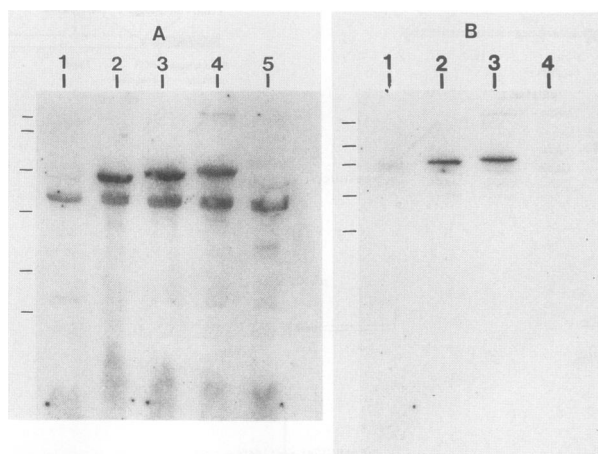
FIG. 9. Western blot showing expression of LamB-B subunit fusions under the expression of modified, synthetic β-lactamase promoter. (A) Host strain *E. coli* pop6510; the bands are revealed with polyclonal anti-LamB antiserum. The Bio-Rad molecular mass marker positions are indicated on the left, and from top to bottom they are 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa. Lanes: 1, pop6510; 2, pSU116 (β-lactamase promoter-*lamB*); 3, pSU119 (β-lactamase promoter-*lamB-stx17B*); 4, pSU118 (β-lactamase promoter-*lamB-stx27B*); 5, pSU117 (β-lactamase promoter-*lamB-stxB*). (B) Host *S. typhimurium aroA* strain SL3261; the bands are revealed with polyclonal B subunit antiserum. The Sigma molecular mass marker positions are indicated on the left, and from top to bottom they are 84.0, 58.0, 48.5, 36.5, and 26.6 kDa. Lanes: 1, pSU116; 2, pSU119; 3, pSU118; 4, pSU117.

would not express the foreign antigen. In vitro-inducible promoters also result in considerable variation of expression, rendering quantitative studies of the amount of antigen delivered during immunization and data on the reproducibility of antibody response very difficult. The batch-to-batch variations observed in our study were considerable. It was therefore desirable to express the hybrid proteins with either a moderately active constitutive promoter or the iron-regulated aerobactin promoter which presumably is induced in vivo in the iron-limiting environment of the intestinal mucosa. This was achieved in our study by the use of a synthetic, modified β-lactamase promoter, which provides moderate-level constitutive expression, and the aerobactin promoter which is induced at low iron concentrations, such as is found in the tissues of animal hosts. With these promoters, it was possible to culture hybrid *S. typhimurium aroA* bacterial cells (except for those expressing the LamB-StxB hybrid protein) to normal cell densities.

The immunization results showed that irrespective of the promoter used, significant B subunit-specific antibody responses were obtained in the intestinal fluid (secretory IgA) and in the serum (IgG and IgM). This result is expected, since in Western blots it was observed that all three promoters expressed significant quantities of the hybrid proteins. However, the use of modified β-lactamase and aerobactin promoters significantly increased the stability of the hybrid *Salmonella* strains. B subunit-specific antibody responses increased as the size of the B subunit region expressed in LamB increased, presumably reflecting increasing epitope densities. Higher antibody levels may not necessarily be as critical in protection as adequate levels of antibodies specifically directed against the B subunit epithelial cell-binding site or of antibodies that sterically hinder B subunit interaction with the receptor. In conclusion, of the various fusion proteins constructed and expression systems explored, the Stx27B hybrid expressed from the β-lactamase or aerobactin promoter is most suitable. Integration of these constructs into the chromosome of the host strain should lead to a reduction in the amount of protein expressed and thereby alleviate any residual toxicity to the host strain. This should enable the preparation of inocula of healthy live cells. Any of the two systems mentioned above could be incorporated into candidate vaccine strains designed to elicit immune re-

pears to be adequate for the expression of hybrid proteins for immunization by the i.p. or intravenous route, since small doses of live bacteria (10^5 to 10^6 cells) are required, compared with oral immunization, for which 10^8 to 10^9 live cells are needed. Upon induction of the *tac* promoter, however, the level of expression of the hybrid proteins was too high and proved toxic to the cells, preventing maximum live-cell density of 10^7 bacteria per ml from being attained. An additional drawback of the *tac* promoter is that it requires in vitro induction with IPTG; in vivo, the antigen carrier strain
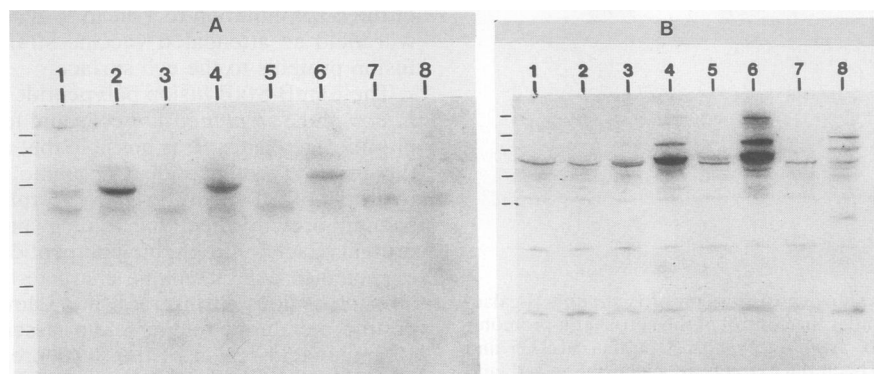

FIG. 10. Western blot showing expression of LamB-B subunit fusions under the expression of the aerobactin promoter. The host strain is *E. coli* pop6510. Panels A and B have been developed with polyclonal LamB antiserum and polyclonal B subunit antiserum, respectively. Paired lanes show uninduced culture and culture induced with dipyridyl, respectively. Lanes: 1 and 2, pSU121 (aerobactin promoter-*lamB*); 3 and 4, pSU124 (aerobactin promoter-*lamB-stx17B*); 5 and 6, pSU123 (aerobactin promoter-*lamB-stx27B*); 7 and 8, pSU122 (aerobactin promoter-*lamB-stxB*). For both gels, the molecular mass marker positions are shown on the left. From top to bottom, they are 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa (A) and 84.0, 58.0, 48.5, 36.5, and 26.6 kDa (B).
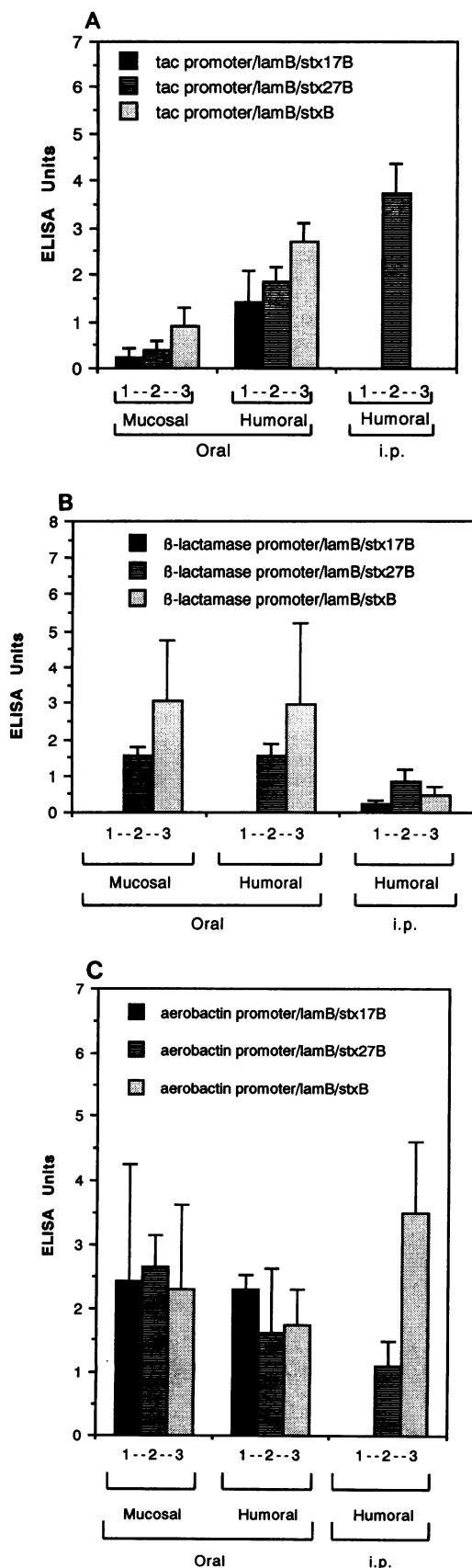
FIG. 11. Serum IgG and IgM and mucosal IgA B subunit-specific responses after oral and i.p. immunizations of BALB/c mice with various *S. typhimurium aroA* hybrid strains expressing LamB-B subunit proteins. Each bar represents the mean of ELISA units determined from four mice. Standard deviation bars are shown. Serum and mucosal B subunit responses were zero for control animals, and therefore, negative controls have not been shown on the graphs. (A) B subunit responses with *S. typhimurium aroA* strain (SL3261) carrying plasmids pSU112 (*tac* promoter-*lamB-stxB*), pSU113 (*tac* promoter-*lamB-stx27B*), pSU114 (*tac* promoter-*lamB-stx17B*), and pAJC264 (*tac* promoter-*lamB* and negative control [data not shown for negative control]). (B) B subunit responses with *S. typhimurium aroA* strain (SL3261) carrying plasmids pSU117 (β-lactamase promoter-*lamB-stxB*), pSU118 (β-lactamase promoter-*lamB-stx27B*), pSU119 (β-lactamase promoter-*lamB-stx17B*), and pSU116 (β-lactamase promoter-*lamB* and negative control [data not shown for negative control]). (C) B subunit responses with *S. typhimurium aroA* strain (SL3261) carrying plasmids pSU122 (aerobactin promoter-*lamB-stxB*), pSU123 (aerobactin promoter-*lamB-stx27B*), pSU124 (aerobactin promoter-*lamB-stx17B*) and pSU121 (aerobactin promoter-*lamB* and negative control [data not shown for negative control]).

sponses against *S. dysenteriae* 1 infections in order to also stimulate mucosal and serum antibody responses against the deleterious effects of Shiga toxin.

ACKNOWLEDGMENTS

REFERENCES

1. **Bassford, P., J. Beckwith, K. Ito, C. Kumamoto, S. Mizushima, D. Oliver, L. Randall, T. Silhavy, P. C. Tai, and W. Wickner.** 1991. The primary pathway of protein export in *E. coli*. Cell **65:**367–368.
2. **Bloch, M., and C. Desaymard.** 1985. Antigenic polymorphism of the LamB protein among members of the family *Enterobacteriaceae*. J. Bacteriol. **163:**106–110.
3. **Boulain, J. C., A. Charbit, and M. Hofnung.** 1986. Mutagenesis by random linker insertion into the *lamB* gene of *Escherichia coli* K-12. Mol. Gen. Genet. **205:**339–348.
4. **Brazil, G. M., C. L. Clayton, T. Sekizaki, and K. N. Timmis.** 1988. Development of DNA probes for cytotoxin and enterotoxin genes in enteric bacteria. Experientia **44:**848–853.
5. **Cantey, J. R.** 1985. Shiga toxin—an expanding role in the pathogenesis of infectious diseases. J. Infect. Dis. **151:**766–771.
6. **Charbit, A., J. C. Boulain, A. Ryter, and M. Hofnung.** 1986. Probing the topology of a bacterial membrane protein by genetic insertion of a foreign epitope: expression at the cell surface. EMBO J. **5:**3029–3037.
7. **Charbit, A., and M. Hofnung.** 1985. Isolation of different bacteriophages using the LamB protein for adsorption on *E. coli* K-12. J. Virol. **53:**667–671.
8. **Charbit, A., A. Molla, W. Saurin, and M. Hofnung.** 1988. Versatility of a vector to express foreign polypeptides at the surface of gram-negative bacteria. Gene **70:**181–189.
8a.**Clements, J.** Personal communication.
9. **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.

10. **Donohue-Rolfe, A., G. T. Keusch, C. Edson, D. Thorley-Lawson, and M. Jacewicz.** 1984. Pathogenesis of *Shigella* diarrhoea. IX. Simplified high yield purification of *Shigella* toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies. J. Exp. Med. **160:**1767–1781.

11. **Endo, Y., K. Tsurugi, T. Yutsudo, Y. Takeda, T. Ogasawara, and K. Igarashi.** 1988. Site of action of Vero toxin (V2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. Eur. J. Biochem. **171:**45–50.

12. **Fontaine, A., J. Arondel, and P. J. Sansonetti.** 1988. Role of Shiga toxin in the pathogenesis of bacillary dysentery, studied by using a Tox⁻ mutant of *Shigella dysenteriae* 1. Infect. Immun. **56:**3099–3109.

13. **Fontaine, A., J. Arondel, and P. J. Sansonetti.** 1990. Construction and evaluation of live attenuated vaccine strains of *Shigella flexneri* and *Shigella dysenteriae* 1. Res. Microbiol. **141:**907–912.

14. **Hanahan, D.** 1983. Studies in transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**577–580.

15. **Harari, I., and R. Arnon.** 1990. Carboxy-terminal peptides from the B subunit of Shiga toxin induce local and parenteral protective effect. Mol. Immunol. **27:**613–621.

16. **Harari, I., A. Donohue-Rolfe, G. Keusch, and R. Arnon.** 1988. Synthetic peptides of Shiga toxin B subunit induce antibodies which neutralize its biological activity. Infect. Immun. **56:**1618–1624.

17. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291:**238–239.

18. **Hopp, T. P., and K. R. Woods.** 1981. Prediction of protein determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA **78:**3824–3828.

19. **Hopp, T. P., and K. R. Woods.** 1983. A computer program for predicting protein antigenic determinants. Mol. Immunol. **20:**483–489.

20. **Jacewicz, M., H. Clausen, E. Nudelman, A. Donohue-Rolfe, and G. T. Keusch.** 1986. Pathogenesis of *Shigella* diarrhoea. XI. Isolation of *Shigella* toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriosylceramide. J. Exp. Med. **163:**1391–1404.

21. **Jackson, M. P.** 1990. Structure-function analyses of Shiga toxin and the Shiga-like toxins. Microb. Pathog. **8:**235–242.

22. **Karmali, M. A.** 1989. Infection by verocytotoxin-producing *Escherichia coli*. Clin. Microbiol. Rev. **2:**15–38.

23. **Kearney, J. F., A. Radbruch, B. Liesegang, and K. Rajewsky.** 1979. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreted hybrid cell lines. J. Immunol. **123:**1548–1550.

24. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.

25. **Lindberg, A. A., J. E. Brown, N. Stromberg, M. Westling-Ryd, J. E. Schultz, and K. A. Karlsson.** 1987. Identification of the carbohydrate receptor for Shiga toxin produced by *Shigella dysenteriae* 1 type 1. J. Biol. Chem. **262:**1779–1785.

26. **Lorenzo, V. d., S. Wee, M. Herrero, and J. B. Neilands.** 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. J. Bacteriol. **169:**2624–2630.

27. **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.

28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

29. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

30. **Mills, S. D., T. Sekizaki, M. I. Gonzalez-Carrero, and K. N. Timmis.** 1988. Analysis and genetic manipulation of *Shigella* virulence determinants for vaccine development. Vaccine **6:**116–122.

31. **Mills, S. D., and K. N. Timmis.** 1988. Genetics of O-antigen polysaccharide biosynthesis in *Shigella* and vaccine development, p. 21–39. *In* F. C. Cabello and C. Pruzzo (ed.), Bacteria, complement and the phagocytic cell. Springer Verlag, Berlin.

32. **Milton, D. L., M. L. Napier, R. M. Myers, and J. K. Hardman.** 1986. *In vitro* mutagenesis and overexpression of the *Escherichia coli trp* A gene and the partial characterization of the resultant tryptophan synthase mutant β-subunits. J. Biol. Chem. **261:**16604–16615.

33. **O'Brian, A. D., and R. K. Holmes.** 1987. Shiga and Shiga-like toxins. Microbiol. Rev. **51:**206–220.

34. **O'Goara, P., D. Maskell, D. Coleman, M. Cafferkey, and G. Dougan.** 1989. Cloning and characterization of the *serC* and *aroA* genes of *Yersinia enterocolitica*, and construction of an *aroA* mutant. Gene **84:**23–30.

35. **Olsnes, S., R. Reisbig, and K. Eiklid.** 1981. Subunit structure of *Shigella* cytotoxin. J. Biol. Chem. **256:**8732–8738.

36. **Palva, E. T., and P. Westermann.** 1979. Arrangement of the maltose-inducible major outer membrane proteins, the bacteriophage lambda receptor in *Escherichia coli* and the 44K protein in *Salmonella typhimurium*. FEBS Lett. **99:**77–80.

37. **Richardson, S. E., M. A. Karmali, L. E. Becker, and C. R. Smith.** 1988. The histopathology of the haemolytic uremic syndrome associated with verocytotoxin-producing *Escherichia coli* infections. Hum. Pathol. **19:**1102–1108.

38. **Roth, J., M. Bendayan, E. Carlemalm, M. Villiger, and M. Garavito.** 1981. Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. J. Histochem. Cytochem. **29:**663–669.

39. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. Science **239:**487–491.

40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

41. **Saxena, S. K., A. D. O'Brien, and E. J. Ackerman.** 1989. Shiga toxin, Shiga-like toxin II variant, and Ricin are all single-site RNA N-glycosidases of 28S RNA when microinjected into *Xenopus* oocytes. J. Biol. Chem. **264:**596–601.

42. **Schägger, H., and G. V. Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.

43. **Schauder, B., H. Blöcker, R. Frank, and J. E. G. McCarthy.** 1987. Inducible expression vectors incorporating the *Escherichia coli atp* E translation initiation region. Gene **52:**279–283.

44. **Schülein, K., and R. Benz.** 1990. LamB (maltoporin) of *Salmonella typhimurium*: isolation, purification and comparison of sugar binding in LamB of *Escherichia coli*. Mol. Microbiol. **4:**625–632.

45. **Sekizaki, T., S. Harayama, G. M. Brazil, and K. N. Timmis.** 1987. Localization of *stx*, a determinant essential for high-level production of Shiga toxin by *Shigella dysenteriae* serotype 1, near *pyrF* and generation of *stx* transposon mutants. Infect. Immun. **55:**2208–2214.

46. **Sekizaki, T., and K. N. Timmis.** 1988. Genetics of Shiga toxin and Shiga-like toxins. Zentralbl. Bakteriol. **17**(Suppl.):177–186.

47. **Slot, J. W., and H. J. Geuze.** 1981. Sizing of protein A colloidal gold probes for immuno-electron microscopy. J. Ultrastruct. Res. **26:**31–43.

48. **Strockbine, N. A., M. P. Jackson, L. M. Sung, R. K. Holmes, and A. D. O'Brien.** 1988. Cloning and sequencing of the genes for Shiga toxin from *Shigella dysenteriae* type 1. J. Bacteriol. **170:**1116–1122.

49. **Swidersky, U. E., H. K. Hoffschulte, and M. Müller.** 1990.

Determinants of membrane-targeting and transmembrane translocation during bacterial protein export. EMBO J. 9:1777–1785.

50. Szmelcman, S., and M. Hofnung. 1975. Maltose transport in E. coli K-12. Involvement of the bacteriophage lambda receptor. J. Bacteriol. 124:112–118.

51. Tesh, V. L., and A. D. O'Brien. 1991. The pathogenic mechanisms of Shiga toxin and the Shiga-like toxins. Mol. Microbiol. 5:1817–1822.

52. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119.