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. BRAHMHBATT,† JÜRGEN WEHLAND, MANFRED ROHDE, AND KENNETH N. TIMMIS

Department of Microbiology, GFB, National Research Centre for Biotechnology, Braunschweig, Germany

Received 24 February 1992/Accepted 22 May 1992

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 antidysestency 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 (Mr = 32,000) and a receptor-binding B subunit (Mr = 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 inflammation 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 antidysestency 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

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

* Corresponding author.
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 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 used as plasmid donors. Plasmid pGC1 (22), with the *lamB* gene under the control of the tac promoter, was a gift from J. E. G. McCarthy, and *E. coli* araA 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 Mutagen 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 Schagger and Jagow (42). SDS-PAGE precasted 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 (OD560) of 0.4 and incubated further until the OD560
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, 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 dithiorthreitol, 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 Boulan 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 pLpX 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 8.0, 6 M guanidinethiocetic acid, 6 M urea, 2% SDS, 0.1% 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 electrophorocloater, 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 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 colloidal-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 10^8 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 immunofluorescence microscopy with a Zeiss microscope. Oral immunization was carried out essentially as described by Clements and colleagues (9). Briefly, for primary immunization with *S. typhimurium* *aroA* strains expressing various Lam-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^{10} CFU/ml. A 0.1-mL cell suspension was used for oral immunization. Cells were further diluted to 10^{8} 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 supernatant was used for oral 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 *SspI* 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 EcoRI site of plasmid pJLA503. The resulting plasmid, pSU108, carries the *stxB* gene under the control of the *lambda* *pL*, and *pR* promoters (in the native *stxB* 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 assumed that the higher-molecular-weight polypeptide was the mature 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 was expected to release the periplasmic contents, the pellet and supernatant samples were electrophoresed on SDS-PAGE. As expected, the pellet contained the high-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 antisera 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, StxBMbl1 and StxBMbl2, 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 StxBMbl1).

**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 this portion of the Shiga toxins is required for the high lethality 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 form "stop transferer" 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 *BamH*I and *BglII* sites at appropriate positions in *stxB* to permit excision of precise fragments of *stxB* and their insertion into the *BamH*I 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 *BamH*I-*SalI* fragment carrying the entire *stxB* gene from plasmid pSU108 (Fig. 1) was subcloned between the *BamH*I-*SalI* 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 *BamH*I 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 *BglII* 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 *BamH*I and *BglII* sites flanking the *stxB* structural gene. A third oligonucleotide, StxB-3 (Fig. 3),
which carries a BglII 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 BamHI site as described for phagemid pSU110 and a BglII 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 BamHI-BglII, and the B subunit inserts were subcloned into the BamHI site of plasmid pAJC264. Subclones 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 BamHI and AccI, blunt ended with DNA polymerase (Klenow enzyme), and religated. This resulted in plasmid pSU114 (Fig. 1), which carries the 17-amino-acid region

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.
fused to LamB (designated lamB-stx\text{17B}). Each of these plasmids was transformed into the \textit{E. coli} host strain pop6510 and the SL3235 \textit{aroA} strain of \textit{S. typhimurium}. Prior to transformation of SL3235, the plasmids were first passaged through the restriction-negative \textit{S. typhimurium} SL5283.

Western blotting analyses of the expression of the LamB-B subunit hybrid proteins in \textit{E. coli} K-12 and in \textit{S. typhimurium} \textit{aroA} SL3235. Whole-cell extracts were prepared with and without IPTG induction from \textit{E. coli} pop6510 and \textit{S. typhimurium} SL3235 carrying plasmids pSU112 (lamB-stx\text{B}), pSU113 (lamB-stx\text{27B}), pSU114 (lamB-stx\text{17B}), 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 \textit{E. coli} and \textit{S. typhimurium} hosts were similar, and hence only the data for the \textit{E. coli} host are shown.

Immunoelectron microscopy analysis of the expression of LamB-B subunit hybrid proteins in various \textit{E. coli} and \textit{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

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>DNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>StxB-N</td>
<td>5'- AATCAAGGGGCTGGATCCGCCACTTGTCTGA 3'</td>
</tr>
<tr>
<td></td>
<td>BamHI</td>
</tr>
<tr>
<td>StxB-C</td>
<td>5'- CTGAGTATCTGGAGATCTCGAAATAC 3'</td>
</tr>
<tr>
<td></td>
<td>IlaAmp</td>
</tr>
<tr>
<td>StxB-3</td>
<td>5'- TGTTGTTAAATGATCTTATGCA 3'</td>
</tr>
<tr>
<td></td>
<td>BamHI</td>
</tr>
<tr>
<td>LamB-8</td>
<td>5'- CTCAGGAGATCTTAATGATGATTACTCTGCT 3'</td>
</tr>
<tr>
<td>LamB-9</td>
<td>5'- GCAACATCGGATTTTCCAG 3'</td>
</tr>
</tbody>
</table>

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 \textit{E. coli} pop6510. Heat-denatured bacterial extracts were electrophoresed on SDS-PAGE. The figure shows expression in \textit{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-StxB 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, 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 (SL3235) cells (Fig. 5B, b, c, and d). These results raised the question of whether another feature of the araA 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 ara genes), SL3261 (another S. typhimurium araA mutant), and AB2829 (an E. coli K-12 araA strain). The hybrid proteins 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 araO, and S. typhimurium SL5283, the LamB protein was detected on the bacterial cell surface, whereas neither of the S. typhimurium araA 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 the araA defect is not responsible for the membrane export defect since the E. coli araO strain also exported the LamB hybrids to the outer membrane. It is likely, therefore, that the S. typhimurium araA 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 araA 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 araA 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 araO, and S. typhimurium (SL5283) cells expressing the Lamb-StxB17B 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 araA 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 10^9 live S. typhimurium araA 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 EcoRI site upstream of the −35 region and a BamHI site following the ATG translation start codon, (ii) XhoI and NdeI 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 EcoRI-BamHI fragment in plasmid pcon1 (Fig. 7) was replaced with the EcoRI-BamHI 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 BglII-ClaI PCR fragment (Fig. 3; oligonucleotides LamB-8 and LamB-9 used for PCR) and was used to replace the BamHI-ClaI fragment of plasmid pSU208 (Fig. 7). The resulting plasmid, pSU115, was cleaved with EcoRI-ClaI, and the β-lactamase promoter (including the PCR fragment) was used to replace the corresponding EcoRI-ClaI fragments in plasmids pAJC264 (lamB), pSU112 (lamB-stxB), pSU113 (lamB-stx27B), and pSU114 (lamB-stx27B), 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 BamHI-BglII stxB fragment from plasmid pSU110 (Fig. 7) was inserted into the BamHI site of plasmid pcon1 to give plasmid pSU207, with the ATG initiating codon upstream of the BamHI site. The BglII-ClaI PCR fragment was subcloned into the BamHI-ClaI sites of plasmid pSU207 to give plasmid pSU120. The EcoRI-ClaI fragment from plasmid pSU120 was used to replace the corresponding fragments of plasmids pAJC264,
ANTI-SHIGA TOXIN COMPONENT OF DYSENTERY VACCINE

VOL. 60, 1992

P. 3353

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>LamB-B subunit fusion</th>
<th>Antibody*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anti-B subunit polyclonal</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-12 pop6510</td>
<td>LamB</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LamB-Stx17B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>LamB-Stx27B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>LamB-StxB</td>
<td>–</td>
</tr>
<tr>
<td>aroA AB2826</td>
<td>LamB</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LamB-Stx17B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>LamB-Stx27B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>LamB-StxB</td>
<td>–</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aroA SL3235</td>
<td>LamB</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LamB-Stx17B</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LamB-Stx27B</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LamB-StxB</td>
<td>–</td>
</tr>
<tr>
<td>aroA SL3261</td>
<td>LamB</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LamB-Stx17B</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LamB-Stx27B</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LamB-StxB</td>
<td>–</td>
</tr>
<tr>
<td>SL5283</td>
<td>LamB</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LamB-Stx17B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>LamB-Stx27B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>LamB-StxB</td>
<td>–</td>
</tr>
</tbody>
</table>

* 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.

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 EcoRI, XhoI, NdeI, and BamHI restriction enzyme sites are also indicated.

**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-Stx27B 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

![Figure 6](image-url)
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 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-StxB17B and LamB-StxB27B 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-
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 $10^9$ cells) are required, compared with oral immunization, for which $10^9$ to $10^10$ 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 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 $\beta$-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 araA 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 $\beta$-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 StxB hybrid expressed from the $\beta$-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-
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-stx17B), 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-stx17B), 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-stx17B), pSU123 (aerobactin promoter-lamB-stx27B), pSU124 (aerobactin promoter-lamB-stx17B) and pSU121 (aerobactin promoter-lamB and negative control [data not shown for negative control]).

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

ACKNOWLEDGMENTS

We thank Singh Chhatwal for assistance with FPLC purification of B subunit and J. E. G. McCarthy for guidance in the design of the synthetic, modified β-lactamase promoter and for synthesis of the oligonucleotides. We also appreciate the technical assistance provided by Silke Fisher.

We gratefully acknowledge the Deutsche Forschungsgemeinschaft (SFBI292 [Gastrointestinal Barriere]) for supporting this work. K.N.T. was additionally supported by the Fonds der Chemischen Industrie.

REFERENCES


8a. Clements, J. Personal communication.


