

Construction of a Plasmid for Expression of Foreign Epitopes as Fusion Proteins with Subunit B of *Escherichia coli* Heat-Labile Enterotoxin

FLORIAN SCHÖDEL* AND HANS WILL

Max-Planck-Institut für Biochemie, Martinsried, Federal Republic of Germany

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A novel vector (pFS2.2) for high-level expression of fusion polypeptides with the nontoxic subunit B (LT-B) of *Escherichia coli* heat-labile enterotoxin in *Escherichia coli* and salmonellae is presented. It carries the complete coding sequence of LT-B under *lac* promoter control and a universal polylinker site for the in-frame insertion of foreign genes at the LT-B gene 3' end. By using this vector, fusion proteins comprising parts of the human or woodchuck hepatitis B virus surface and nucleocapsid antigens are expressed in *E. coli* and salmonella.

The nontoxic subunit B (LT-B) of *Escherichia coli* heat-labile toxin is secreted into the periplasm and assembles to holotoxin oligomers with the adenylate cyclase-activating subunit A in a molar A-to-B ratio of 1 to 5 (10). A signal sequence of 21 amino acids is cleaved, resulting in a molecular size of 11.8 kilodaltons (kDa) for the mature monomeric LT-B molecule (3, 6). In the host intestine, LT-B binds to ganglioside GM₁ and elicits both a mucosal immunoglobulin A and a systemic immunoglobulin G response when applied orally (2, 4). This property has been exploited in the construction of a bivalent oral vaccine against *E. coli* and cholera enterotoxin and typhoid fever which consists of recombinant salmonellae that express LT-B (2). Attenuated recombinant salmonellae expressing a circumsporozoite antigen have recently been used as an oral vaccine and provide protection against *Plasmodium berghei* infection in mice (22). Because LT-B is itself a strong immunogen and can be expressed to high levels in attenuated *Salmonella* strains under *lac* promoter control (4), we constructed a vector allowing the translational fusion of foreign epitopes with the carboxy terminus of LT-B (Fig. 1, pFS2.2).

The LT-B coding sequence was excised from pJC217 (a gift of John Clements) (4) as a 0.8-kilobase *Hind*III fragment (all restriction enzymes were from Boehringer GmbH, GIBCO Laboratories, or New England BioLabs; standard procedures were followed as described in reference 14) and purified by agarose gel electroelution (7). After removal of the translational stop codon of LT-B by *Mae*I digestion and further gel purification, the *Hind*III-*Mae*I fragment was added to pUC19 (28) digested with *Hind*III and *Kpn*I and a 100-fold molar excess of two complementary synthetic oligonucleotides with *Kpn*I and *Mae*I sticky ends, ligated with T4-ligase, and transformed into JM83 (15). The sequence of the coding-strand synthetic oligonucleotide was 5'-TAC CCTCAG GATCCGATA TCTAATTA TTAGTAC-3', providing 3' translational stop codons in all three reading frames. The nucleotide sequence of the universal polylinker at the 3' end of the LT-B coding sequence was confirmed, as were all inserted DNA sequences mentioned below, by DNA sequencing (23) (α -³⁵S]dATP was purchased from Amersham-Buchler; Klenow and Sequenase were purchased from Boehringer and United States Biochemical Corp., respec-

tively). By using this polylinker and in one case the *Sma*I site of LT-B (pFS3), different segments of human (HBV) and woodchuck (WHV) hepatitis B virus surface or nucleocapsid coding sequences were fused to the LT-B coding sequence (Fig. 2).

We chose the hepatitis B virus because HBV infection still constitutes one of the major worldwide health concerns; there are over 200 million chronic carriers, of which a large percentage succumb to hepatocellular carcinoma (1). Despite the existence of a safe and effective plasma-derived vaccine and a recombinant vaccine (21), an eradication strategy continues to be hampered by the cost of the existing vaccines, which makes it unavailable to the countries with the highest incidence of HBV. With the DNA sequence available (9) and some protective epitopes of the envelope proteins defined (11, 16, 18), the construction of recombinant LT-B-virus fusion proteins later to be evaluated for their protective capacity seemed feasible. A hepadnavirus (WHV) closely related to HBV causes hepatic disease in the American woodchuck *Marmota monax* (for a review, see reference 23a). Because the only animal model available for HBV infection is the chimpanzee, we constructed similar LT-B-virus expression vectors for the WHV system to be able to test the efficacy in a less delicate and costly species.

For insertion of HBV surface or core genes, appropriate fragments were isolated from plasmid pTW3093 or pTW346 (courtesy of T. Weimer, unpublished results), both containing HBV DNA of subtype ayw (9). WHV DNA was isolated from pSPW1S (T. Weimer, unpublished results) or pUWT1 (F. Schödel, unpublished results), both harboring WHV1 DNA (8) as a monomer or head-to-tail tandem, respectively. For the construction of pFS17, two complementary synthetic oligonucleotides coding for an HBV pre-S2 B-cell epitope site (amino acids 133 to 151; amino terminus of pre-S2 at amino acid 120) (11, 16) were inserted into pFS2.2-*Sau*I-*Bam*HI. The coding-strand sequence was 5'-TCAG GACCC GCGTGTTCGT GGTCTGTACT TCCCGGCTGG TGTTCCCTCC TCCGGTACCG TT-3'. *E. coli* JM83 and JM105 were used as plasmid hosts and for the study of expression in *E. coli* (15, 28). For expression of LT-B or LT-B fusion proteins, these strains were grown in ML medium (2) (0.5 g of glucose/liter) in the presence of ampicillin (100 µg/ml) (all chemicals were obtained from Merck & Co., Inc., Sigma Chemical Co., or Fluka). *Salmonella*

* Corresponding author.

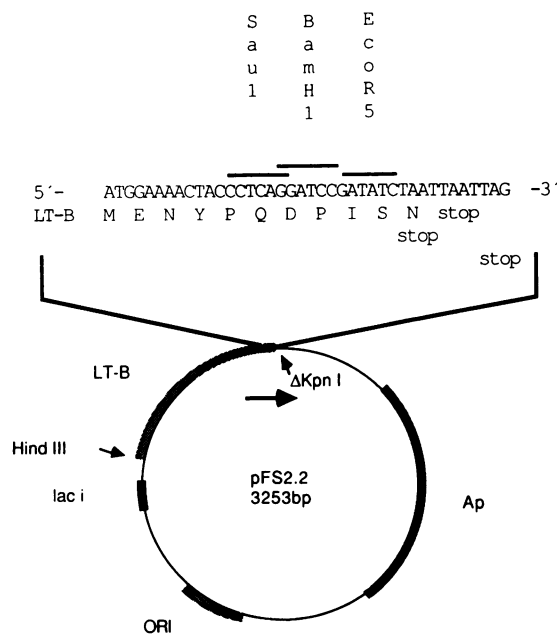


FIG. 1. Structure of the expression plasmid pFS2.2 and restriction map of the universal polylinker site. LT-B coding sequence and polylinker are inserted between the *Hind*III and *Kpn*I sites of pUC19 (28). The carboxy-terminal three amino acids of LT-B (MEN) are followed by the polylinker translation sequence.

strains SL5235, SL5283, and SL1438 (kindly provided by Bruce Stocker) (24) were grown in the presence of 2,3-dihydroxybenzoic acid (1 μ g/ml). Transformation of bacteria with plasmids was performed by the conventional CaCl_2 method (5), with the exception of salmonellae, which were transformed either with an extended heat shock period (30 min at 37°C) or by the method of Lederberg and Cohen (13). Plasmids from *E. coli* were CsCl purified and sequentially transferred to SL1438 via one of the $r^{-}m^{+}$ strains SL5235 or SL5283 (B. Stocker, personal communication). Expression of LT-B and LT-B-virus fusion proteins was studied in *E. coli* (Fig. 3) and salmonellae (Fig. 4) by immunoblotting of bacterial extracts and by GM_1 enzyme-linked immunosorbent assay (data not shown; modification of methods in references 17 and 25) of supernatants, osmotic shock fractions, and lysates. For immunoblotting, bacterial cell pellets of overnight cultures were lysed in Laemmli buffer (12) and heat denatured. The proteins were separated by 12.5 or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and visualized by antibody and ^{125}I -protein A autoradiography (Fig. 3 and 4A and B) or phosphatase-coupled second antibody (Fig. 4C). Antibodies were raised against purified LT-B (kind gift of John Clements) or purified recombinant viral proteins (27) in rabbits. A monoclonal antibody against the dominant HBV pre-S2 epitope was kindly provided by M. Mayumi (19), and anti-peptide antibodies which recognize virus surface antigens were provided by A. R. Neurath and D. R. Milich.

The LT-B coding sequence in pFS2.2 is under the control of the *lac* promoter but retains the DNA sequences in the vicinity of the LT-B ATG that may lead to LT-B translation in at least 5 M excess over that of LT-A from the same polycistronic mRNA in vivo (6). In the salmonellae tested, the *lac* promoter is constitutively active. Even in a *lacI^a* *E. coli* strain (JM105), translation products were found without isopropyl- β -D-thiogalactopyranoside induction (data not

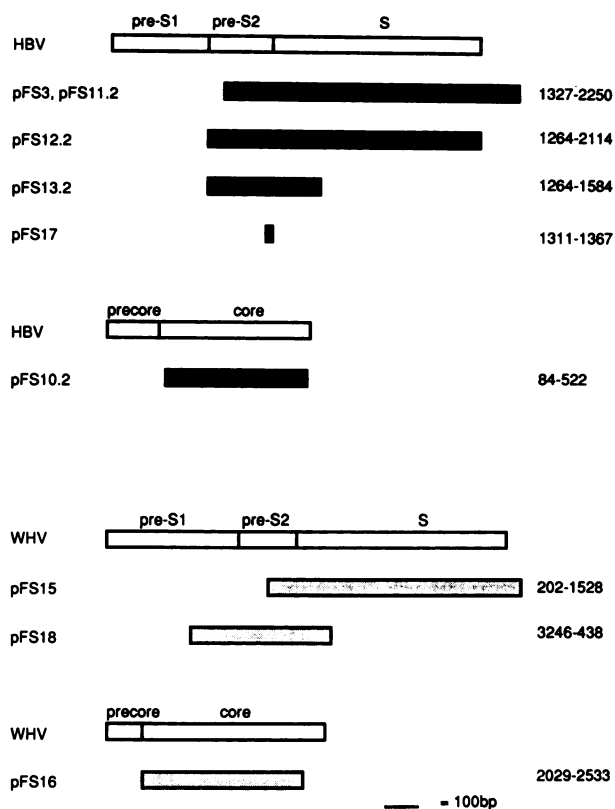


FIG. 2. Map of HBV and WHV genes fused to the LT-B gene. All plasmids represent in-frame gene insertions into the polylinker site of pFS2.2, with the exception of pFS3, in which the HBV sequence is inserted into the *Sma*I site of LT-B. The open reading frames coding for the large (pre-S1), middle (pre-S2), and small (S) surface antigens and the nucleocapsid or core antigens on the (circular) minus-strand DNA of HBV and WHV are schematically illustrated; positions of their (probable) translational start and stop codons are indicated by vertical bars (for a detailed description, see reference 23a). Numbers on the right give the nucleotide positions on the HBV (20) and WHV (8) genomes of the gene fragments fused to LT-B. The HBV nomenclature has the A of the core ATG and the WHV nomenclature has the first A of the unique *Eco*RI site as position 1.

shown). The high copy number of pUC19, in relation to repressor molecules, in combination with very efficient initiation of translation at the LT-B AUG may account for this finding. The complete amino acid sequence of LT-B is conserved, and an additional eight amino acids are added to the carboxy terminus by the polylinker (Fig. 1). LT-B expressed from pFS2.2 retained GM_1 binding, since it was readily detected by GM_1 enzyme-linked immunosorbent assay (results not shown) by using our polyclonal rabbit anti-LT-B or two monoclonal antibodies (26) (kindly provided by A. M. Svennerholm and J. Holmgren) and elicited an immunoglobulin G response when fed to mice in SL1438 (results not shown). Its predicted molecular size is 12.7 kDa compared with 11.7 kDa for mature monomeric unmodified LT-B, consistent with its retarded migration pattern observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3A and 4). The level of expression of LT-B from pFS2.2 roughly equalled that of pJC217 (4) in SL1438 (e.g., Fig. 4A) and was estimated by immunoblotting and enzyme-linked immunosorbent assay data to be in the range of 10 to 15 μ g per ml of overnight culture (approximately 2 \times

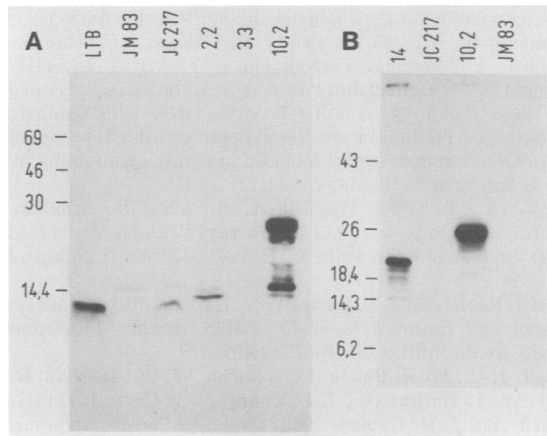


FIG. 3. Immunoblots of bacterial protein extracts from *E. coli* JM83 overnight cultures (approximately 225 μ l per lane). Lane LT-B contained 2 μ g of purified LT-B. Plasmid designation as in Fig. 2 (e.g., 2.2 = pFS2.2). JM83 is a strain without plasmid. Plasmid pJC217 (4), expressing native LT-B, was added as a reference. (A) LT-B epitopes on proteins expressed in *E. coli*. Lane 3.3, JM83(pFS3). Antibody was rabbit α -LT-B. (B) Core epitopes on proteins expressed in *E. coli*. Antibody was rabbit α -HBc. Plasmid pFS14 expresses HBc of authentic size regulated by a *tac* promoter (F. Schödel, unpublished results) and is given as a size control. Molecular sizes in kilodaltons are shown to the left of each panel.

10^9 CFU). Expression of fusion polypeptides differed between *E. coli* and attenuated *Salmonella dublin* SL1438. Peptides comprising the entire pre-S2 region and the amino terminus of HBs-Ag (pFS13.2) and a dominant pre-S2 B-cell epitope (pFS17) (11, 16, 18) were detectable both in *E. coli* (results not shown) and in SL1438 (Fig. 4A and C). In contrast, LT-B-HB core fusion proteins (pFS10.2) expressed in *E. coli* in amounts similar to LT-B from pFS2.2 (Fig. 3) remained undetectable in salmonellae (Fig. 4A, lanes 10.21 and 10.22). In addition, the pre-S region and a part of WHV core (pFS18 and pFS16) fused to LT-B were expressed both in *E. coli* (not shown) and in salmonellae (Fig. 4A and B). No fusion proteins were detectable from constructions compris-

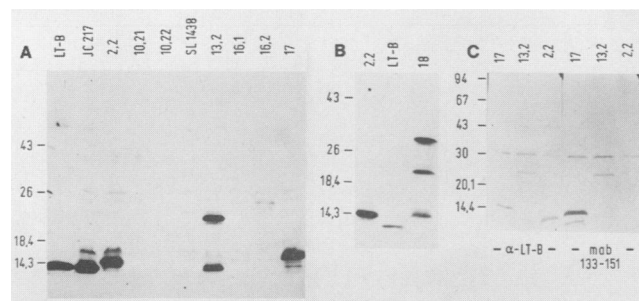


FIG. 4. Immunoblots of bacterial protein extracts of SL1438 overnight cultures. Plasmids are as shown in Fig. 2. SL1438 is a strain without plasmid. 10.21 and 10.22 are two different SL1438(pFS10.2) clones. (A) LT-B epitopes on proteins expressed in *E. coli*. Antibody was rabbit α -LT-B. Lane LT-B contained 10 μ g of purified LT-B. (B) LT-B epitopes on a WHV pre-S fusion protein expressed in SL1438. Antibody was rabbit α -LT-B. Lane LT-B contained 2 μ g of purified LT-B. Approximately 225 μ l of overnight culture was used per lane. (C) Pre-S2 epitopes compared to LT-B epitopes on pFS2.2, pFS13.2, and pFS17. Extracts of approximately 70 μ l of overnight culture were used per lane. Antibodies were rabbit α -LT-B or α -pre-S2 monoclonal antibody (19).

ing the complete coding sequence of the small surface antigen of HBV or WHV (pFS3, pFS11.2, pFS12.2, and pFS15).

In all fusion peptides detected by anti-LT-B immunoblot, the presence of the viral epitopes could be confirmed by immunoblotting with the appropriate antiviral antibodies (for examples, see Fig. 3B and 4C). Fusion proteins of the expected molecular sizes were expressed in salmonellae with a similar efficiency (pFS17; expected molecular size, 14.6 kDa), about a two- to fivefold-reduced efficiency (pFS13.2 and pFS18; 23.7 and 30.6 kDa, respectively), or a dramatically reduced efficiency (pFS16.2, 32 kDa) compared with LT-B expression from pFS2.2 (Fig. 4A and B). In most cases two or three distinct immunoreactive bands migrating faster than a fusion protein of the expected size which may correspond to proteolytic products of the fusion proteins were observed as well. Since fusion proteins from pFS13.2 and pFS17 were detectable in the supernatant and in the osmotic shock fraction when tested with antiviral antibodies in a GM₁ enzyme-linked immunosorbent assay (results not shown), we assume that they are also periplasmatically secreted in salmonellae. In preliminary experiments, male BALB/c mice fed recombinant *Salmonella* strain SL1438 (pFS2.2), SL1438(pFS13.2), or SL1438(pFS17) (10^{10} CFU in phosphate-buffered saline via orogastric tube) developed serum anti-LT-B immunoglobulin G after the second inoculation, which could be boosted by additional peroral immunizations (results not shown). The carboxy-terminally modified LT-B expressed by our vectors thus retains the oral immunogenicity of native LT-B (4). In addition, splenic cells reactive to LT-B could be cultured from mice fed recombinant SL1438(pFS2.2), SL1438(pFS13.2), and SL1438 (pFS17) (G. Enders, H. Will, and F. Schödel, manuscript in preparation). Systemic antibodies against the viral sequence could not be detected so far in these mice. Further experiments to determine the immunogenicity of the virus fusion parts by analysis of T- and B-cell responses and protection against virus challenge in woodchucks are in progress.

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