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Oral Immunization of Mice with a Live Recombinant *Yersinia enterocolitica* O:9 Strain That Produces the Cholera Toxin B Subunit

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The 70-kilobase pYV plasmid of *Yersinia enterocolitica* encodes a set of proteins called Yops that are produced during infection. To use *Y. enterocolitica* as a live carrier to present the cholera toxin B (CT-B) subunit to the immune system, we constructed an operon fusion between *ctxB* and the *yop51* gene. This operon fusion was either cloned on an RSF1010-derived plasmid or integrated into the pYV plasmid itself. In *Y. enterocolitica*, both constructions directed the synthesis of free CT-B only under conditions of Yops production, i.e., at 37°C in a medium deprived of Ca^{2+}. Bacteria containing both types of recombinant plasmids were given orally to mice. A serum antibody response against CT-B was detected in both cases. A secretory immunoglobulin A activity specific to CT-B was also observed in the intestinal secretions. According to immunoblot analysis, the serum antibody response was only directed against the polymeric form of the B subunit. The *ctxB* gene was also inserted in frame within *yop51*, giving a chimeric Yop51-CT-B protein that was secreted into the surrounding medium. In this case, however, no antibody response was observed after oral inoculation of mice. This lack of response probably results from the inability of the hybrid protein to assemble into the polymeric form of the B subunit.

Yersinia enterocolitica is an agent of gastrointestinal disease in humans and animals such as small rodents. The most common clinical manifestation is a mild to moderate enterocolitis characterized by diarrhea, fever, and abdominal pain. Among *Y. enterocolitica* strains, those of serotypes O:3 and O:9, commonly isolated in Europe and Japan, are of lower natural virulence than those of serotype O:8, only encountered in North America (1, 14).

Y. enterocolitica enters its host via the oral route. Reaching the intestinal lumen, the bacteria penetrate the lamina propria of the distal ileum where they multiply preferentially in the Peyer's patches. The bacteria are drained into the mesenteric lymph nodes, but they rarely invade deeper organs (47).

Chromosome- and plasmid-encoded factors are involved in the pathogenesis of yersiniosis. Two chromosomal factors, Inv and Ail, could be responsible for the in vivo crossing of the intestinal epithelium (35; for a review, see reference 29). Another chromosomal locus, *yst* (I. Delor and G. Cornelis, submitted for publication), encodes an enterotoxin similar to heat-stable enterotoxin (46). A plasmid of 70 kilobases (kb), called pYV, encodes at least 10 proteins termed Yops that are involved in the next stages of the infection. Yop production only occurs at 37°C in the absence of Ca^{2+} and correlates with a severe growth restriction. The *yop* genes are scattered around pYV and are coordinately regulated by the product of gene *virF*, also present on pYV (11). The Yop proteins are secreted in the culture supernatant (26) by specific factors presumably encoded by the pYV plasmid, but so far unidentified. At present, the structural genes of nine of these Yops have been localized on the plasmid of *Y. enterocolitica* O:9. Mutation in any of these *yop* genes results in a significant increase of the 50% lethal dose for the mouse (4, 12, 13, 22, 37, 44; for reviews, see references 8 and 10).

Since the Yop proteins are produced during infection by *Y. enterocolitica*, we assumed that the insertion of foreign genes downstream from a *yop* promoter would bring about their expression in the host, presumably when the bacteria interact with the immune system. By production of an appropriate protective immunogen, *Y. enterocolitica* could thus be converted into a live recombinant oral vaccine for various diseases. In a previous work, we used β-galactosidase to show that an antigen whose production was regulated by the virulence plasmid of *Y. enterocolitica* elicited a specific antibody response in mice after oral inoculation of the live recombinant bacteria (44). These results prompted us to investigate the potentiality of *Y. enterocolitica* to act as a live carrier for the cholera toxin B subunit.

The CT is a heat-labile 84-kilodalton (kDa) protein composed of two types of subunits, A (27 kDa) and B (11.6 kDa), encoded by a single transcriptional unit, *ctxAB*. Each toxin molecule is composed of one A subunit that activates the adenylate cyclase of the enterocytes. The resultant ion flux changes in the ileum lead to the severe fluid loss characteristic of cholera. Five B subunits associated with a single A subunit facilitate the entry of the A fragment into the host cell by attaching to GM1, the cell surface receptor of the toxin. The B subunit (CT-B) is the immunologically dominant domain of the CT (21).

Here we report the construction of *Y. enterocolitica* strains in which *ctxB* is under the control of the *yop* regulon. We analyzed the production of CT-B in vitro, and we showed that the oral inoculation of mice with recombinant strains elicits plasma immunoglobulin G (IgG) and intestinal secretory IgA responses against CT-B.

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TABLE 1. Plasmids used in this study

Plasmid	Genotype or description	Origin or reference
pBM7	pYVe227 *yop20-7*::Tn*2507*	37
pBM7-4	Derivative of pBM7 containing plasmid pMSV14	This work
pGC1256	pGB63 *yop25-1256*::mini-Mu d1 *lac*	13
pGP704		36
pGV904	pBR322::Tn*732*	
pIC20H		32
pMS1	pTM100 + *Bgl*II-*Xba*I fragment of pYVe227 containing 459 bp of *yop51*	This work
pMS2	pMS1 + *Cla*I fragment from *Vibrio cholerae* containing *ctxB*	This work
pMS4	pKT230 + *Bam*HI-*Sal*I fragment from pMS2 containing the operon fusion *yop51-ctxB*	This work
pMS41	Derivative of pMS4 containing the operon fusion *yop51-ctxB*	This work
pMS44	Derivative of pMS4 containing the fusion gene *yop51-ctxB*	This work
pMSV13	pGP704 + *Eco*RV-*Bgl*II fragment from pMS41 containing the operon fusion *yop51-ctxB*	This work
pMSV14	pMSV13 + *Bam*HI fragment containing the gene for gentamicin resistance	This work
pRIT10810	pBR322 + *Cla*I fragment containing *ctxB*	33
pTM100	pACYC184-*oriT* of RK2	T. Michiels and G. Cornelis, unpublished data
pYVe227	Virulence plasmid of *Y. enterocolitica* W227 serotype O:9	
pYVe439-80	Virulence plasmid of *Y. enterocolitica* 439-80 serotype O:9, undistinguishable by restriction from pYVe227	3

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains 439-80 and W227 are typical *Y. enterocolitica* strains from serotype O:9. Strain W22703 (nalidixic acid resistant) is a restriction mutant (Res^- Mod^+) of W227 isolated earlier in this laboratory (9). *Escherichia coli* S17.1 (43) was used to mobilize plasmid pMS2 into *Y. enterocolitica* W22703. Mobilization of pMS4, pMS41, and pMS44 into *Y. enterocolitica* was promoted by plasmid RP4 during a triple cross as described previously (44). *E. coli* SM10 λ*pir* is the host strain used to maintain pGP704 and its derivatives. The gentamicin resistance gene subcloned in pGV904 was from Tn*732* (39). Plasmids are described in Table 1.

Bacteria were grown in brain heart infusion broth supplemented with 20 mM $MgCl_2$ and 4% (wt/vol) glucose. This medium was supplemented either with 20 mM sodium oxalate or with 5 mM $CaCl_2$. Antibiotics used were ampicillin (50 or 200 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}), gentamicin (20 μg ml^{-1}), kanamycin (25 μg ml^{-1}), nalidixic acid (35 μg ml^{-1}), streptomycin (25 μg ml^{-1}), and tetracycline (10 μg ml^{-1}).

Induction of *yop* regulon and protein analysis. The *yop* regulon was induced as described by Cornelis et al. (13). Supernatant proteins and whole-cell lysates were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting as outlined by Sory and Cornelis (44). The human anti-Yops serum was from an individual convalescing from a *Y. enterocolitica* O:9 infection. Rabbit antiserum against *Y. enterocolitica* was obtained by injection of formalinized yersiniae grown at 37°C in a medium deprived of Ca^{2+}. This serum reacts with all the major Yops except Yop48. Goat antiserum against CT-B was from Calbiochem-Behring (La Jolla, Calif.). The anti-CT-B serum was absorbed with yersiniae grown at 37°C in a medium deprived of Ca^{2+}. Serum (100 μl) was absorbed by overnight incubation with about 2×10^{10} washed bacteria suspended in phosphate-buffered saline (PBS) (50 mM sodium phosphate, 150 mM NaCl, pH 7.4) containing 0.01% (wt/vol) merthiolate. CT-B was purchased from Sigma Chemical Co. (St. Louis, Mo.).

DNA manipulations and analysis. DNA was prepared and analyzed by standard methods (31). DNA restriction enzymes were from Pharmacia (Uppsala, Sweden) and Boehringer GmbH (Mannheim, Federal Republic of Germany). To create deletions by exonuclease BAL 31 (Boehringer), we incubated 1 μg of DNA in 0.02 M Tris hydrochloride (pH 7.2)–0.4 M NaCl–0.012 M $CaCl_2$–0.012 M $MgCl_2$–0.001 M EDTA with 2 units of BAL 31 at room temperature (RT) for 1, 2, and 5 min. The digestion was stopped by the addition of phenol.

DNA was sequenced by the dideoxy-chain termination procedure with the DNA polymerase Sequenase (United States Biochemical, Cleveland, Ohio) as described in reference 11. The fragments to be sequenced were cloned in the phasmid pTZ19R (Pharmacia).

Preparation of RNA and Northern (RNA) blot hybridization. Total cell RNAs were prepared by hot-phenol extraction done by a modified method of Derbyshire et al. (17) as described by Michiels and Cornelis (34). RNAs were analyzed on 1.2% agarose gels containing 16% formaldehyde and blotted on Hybond N membrane (Amersham, Little Chalfont, United Kingdom). Hybridization with nick-translated probes was done at 50°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50 mM phosphate buffer (pH 6.5)–1× Denhardt solution–0.1% SDS–10 μg of salmon sperm DNA ml^{-1}. Membranes were washed three times for 5 min at RT in 2× SSC–0.1% SDS and twice for 15 min in 0.1× SSC–0.1% SDS.

Animal infection. *Y. enterocolitica* strains were inoculated in female BALB/c mice that were 10 weeks old (bred at the University of Louvain, Woluwe, Belgium). Mice were injected intraperitoneally with 0.5 ml of 5-mg ml^{-1} iron dextran (Imferon; Fisons, Leuven, Belgium) on day 1 and with 0.5 ml of 1% (wt/vol) Desferal (CIBA-GEIGY, Brussels, Belgium) the next day. On the same day, 2×10^9 to 5×10^9 live bacteria grown in brain heart infusion broth at RT were given orally or by gastric intubation.

Oral inoculation of C57BL/6 mice (bred at the University of Louvain) was done by gastric intubation of about 4×10^{10} bacteria, without intraperitoneal injection of iron dextran and Desferal. During the experiments, no animal died from *Yersinia* infection.

Collection of serum and plasma. The immunoblots were done with serum. For this purpose, blood was collected from the heart and the serum was stored at −20°C. The enzyme-linked immunosorbent assays (ELISAs) were done on plasma. For this purpose, blood was collected from the retro-orbital plexus with a Pasteur pipette wetted with 10 μl of 2,500 IU of heparin (Novo Industry, Brussels, Belgium) ml^{-1}. Final plasma was frozen at −20°C.

Collection of intestinal secretions. Intestinal secretions were collected by the method of Elson et al. (20). Each mouse was placed on a wire mesh resting on a petri dish containing 3 ml of a solution of 0.1 mg of soybean trypsin inhibitor (Koch-Light Laboratories, Haverhill, England) ml^{-1} in 50 mM EDTA. Four doses of 0.5 ml of lavage solution (25 mM NaCl, 40 mM Na_2SO_4, 10 mM KCl, 20 mM $NaHCO_3$, 48.5 mM polyethylene glycol 4000) were given intragastrically at 15-min intervals. At 30 min after the last dose, the mice were given intraperitoneally 100 μl of pilocarpine at 1 mg ml^{-1}. A discharge of intestinal contents occurred over the next 5 to 10 min. After collection, 3 ml of PBS was added to rinse the petri dish and the sample was transferred to a 10-ml tube, vigorously vortexed, and centrifuged for 10 min at 650 × *g*. A 30-μl portion of 100 mM phenylmethylsulfonyl fluoride in 95% ethanol was added to 3 ml of supernatant. This was further centrifuged at 27,000 × *g* at 4°C for 20 min. A 2-ml sample of this clarified secretion solution was removed and supplemented with 20 μl of 100 mM phenylmethylsulfonyl fluoride in 95% ethanol–20 μl of 1% (wt/vol) sodium azide–100 μl of 70 mg of bovine serum albumin ml^{-1}. The final solution was frozen at −20°C.

Recovery of bacteria from infected mice and study of plasmid content. Groups of three to six mice were sacrificed 3 or 8 days after intragastric inoculation. The distal part of the small intestine (about 10 cm long) was removed aseptically and cut open longitudinally. The intestinal tissue was washed for 5 min in saline containing 0.1% (vol/vol) Triton X-100 (Serva, Heidelberg, Federal Republic of Germany), rinsed rapidly, and homogenized. Bacteria were quantitated by plating on MacConkey agar containing nalidixic acid.

The plasmid content of the bacteria was analyzed by replica plating on tryptic soy agar containing one of the following antibiotics: gentamicin to monitor the stability of pMSV14; kanamycin to check the presence of pBM7 and pGC1256; and streptomycin to test the stability of pMS41 and pMS44.

GM1-ELISA. GM1-ELISA was developed as outlined by Svennerholm and Holmgren (45). Polystyrene plates (96 wells; Greiner, Nurtingen, Federal Republic of Germany) were coated overnight at 4°C with GM1 (Sigma). Each well received 200 ng in 15 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.6). Between every step of the assay, plates were washed three times with PBS containing 0.1% (vol/vol) Tween 20 (Serva). After saturation of the plates with 1% (wt/vol) bovine serum albumin in PBS-Tween for 1 h at RT, 100 ng of purified CT-B was added to each well for 1 h at 37°C. The following stages of the GM1-ELISA varied according to the immunoglobulin class to be assayed.

(i) Measurement of total plasma anti-CT-B immunoglobulins. Doubling dilutions of plasma were done in triplicate (the first dilution was 1/50), and the plates were incubated for 2 h at 37°C. Total mouse immunoglobulins were detected with peroxidase-conjugated immunoglobulins (Dakopatts, Glostrup, Denmark) diluted 1,000-fold in PBS-Tween. To reveal peroxidase-labeled antibodies, 100 μl of a substrate solution containing 0.04% (wt/vol) *o*-phenylenediamine (Sigma) and 0.001% H_2O_2 in 0.1 M citrate–0.2 M phosphate buffer (pH 5) was added. Revelation was stopped after 3 min with 50 μl of 2 M H_2SO_4. The optical density (OD) was read at 492 nm in a Titertek Multiskan PLUS reader (Flow Laboratories, Brussels, Belgium). We defined the titer as the reciprocal of the dilution giving an OD_{492} of 0.3.

(ii) Measurement of plasma anti-CT-B IgG. Serial twofold dilutions (in triplicate) in PBS-Tween of plasma and standard immunopurified anti-CT-B IgG (41) were added, and the plates were left for 2 h at 37°C. Mouse IgGs were detected with specific peroxidase-conjugated immunoglobulins (gamma-chain specific; Sigma). The reaction was stopped after 10 min with 50 μl of 2 M H_2SO_4.

(iii) Measurement of anti-CT-B IgA of intestinal secretions. For the measurement of total IgA in intestinal fluid, plates were coated with purified sheep anti-mouse IgA (alpha-chain specific; Sigma). Serial twofold dilutions (in triplicate) of a mouse IgA standard (MOPC-315 dimeric IgA [25]) and of intestinal secretions were incubated for 2 h at 37°C. They were revealed by peroxidase-conjugated goat anti-mouse IgA (alpha-chain specific; Sigma).

After this, the secretion samples normalized at 1 μg of total IgA ml^{-1} were incubated in the GM1-coated plates for 2 h at 37°C. Mouse IgA antibodies were detected with specific peroxidase-conjugated immunoglobulins (alpha-chain specific) in PBS-Tween. The anti-CT-B IgA was expressed as OD_{492} for 1 μg of total IgA ml^{-1}. For the assay of secretory anti-CT-B IgA, the secretion samples were normalized at 3 μg of total IgA ml^{-1}. They were added to the wells and incubated for 2 h at 37°C. Secretory IgA was detected by incubation for 1 h at 37°C with an immunopurified rabbit anti-mouse secretory component serum (30) diluted 100-fold in PBS-Tween, followed by peroxidase-conjugated swine anti-rabbit immunoglobulins previously absorbed by mouse immunoglobulins. The anti-CT-B secretory IgA level was expressed as OD_{492} for 3 μg of total IgA ml^{-1}.

RESULTS

Construction of hybrid genes or operons between *yop51* and *ctxB*. To express CT-B in *Y. enterocolitica*, we cloned gene *ctxB* behind the promoter of gene *yop51* encoding the 51-kDa Yop (Yop51). A fragment containing the promoter and the first 459 base pairs (bp) of *yop51* was cloned in a mobilizable pACYC184 derivative (Fig. 1). A fragment containing *ctxB* was then inserted behind *yop51*. In the operon fusion obtained, 142 bp containing the 3′ end of *ctxA* separated the truncated *yop51* gene and the start codon of *ctxB*. This plasmid was called pMS2.

To reduce the intervening DNA between *yop51* and *ctxB*, the fragment of pMS2 containing *yop51* and *ctxB* was first subcloned in the RSF1010-derived vector pKT230. The resulting plasmid, pMS4, was linearized at the unique *Xba*I site located between *yop51* and *ctxB*, digested by the exonuclease BAL 31, and religated (Fig. 2). Twenty plasmids with a deletion shorter than 300 bp were transferred into *Y. enterocolitica* W22703(pGC1256) (see below) and tested for CT-B production under conditions of Yop expression.

According to the immunoblot analysis, 11 recombinant plasmids directed the production of free intracellular CT-B. One of them, called pMS41, was selected for further analysis. Strain W22703(pGC1256)(pMS41) released the Yops and an 18-kDa protein reacting with the anti-Yops serum (Fig. 3A). Hence, this 18-kDa protein was assumed to be a truncated protein of Yop51 and called Yop51′. Incubation of this strain at 28 and 37°C in a medium enriched with or

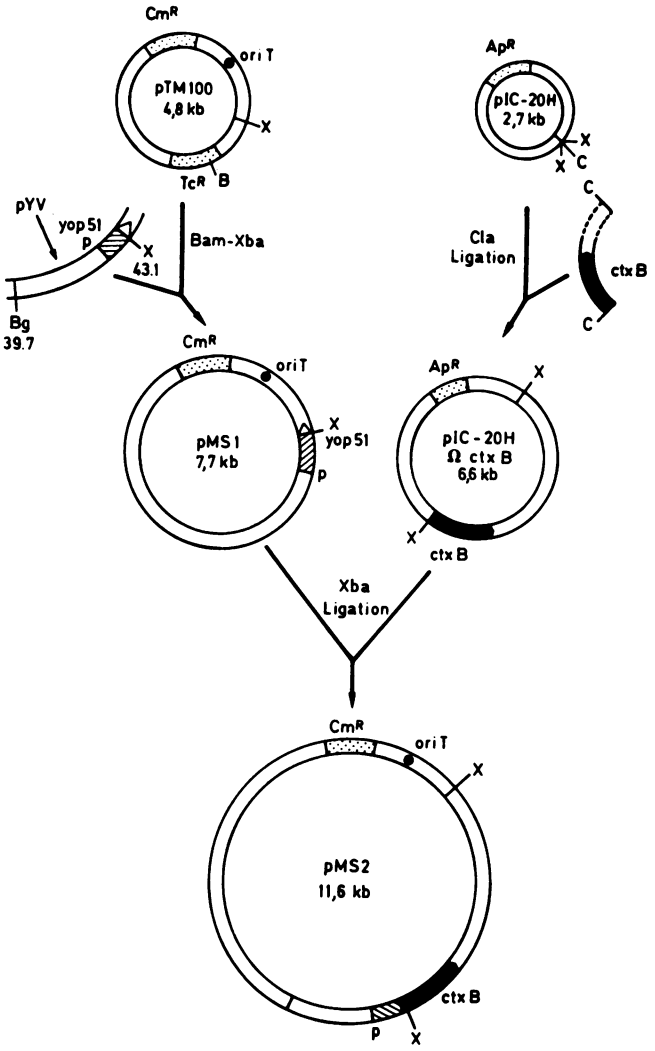

FIG. 1. Construction of pMS2. Plasmid pTM100 is a pACYC184 derivative containing the *oriT* site of RP4. Plasmid pMS1 was first constructed by insertion of a *Bgl*II-*Xba*I (39.7 to 43.1 kb) fragment of plasmid pYVe439-80 in pTM100. This fragment contains the promoter and the first 459 nucleotides of *yop51* (34). In parallel, a 3.9-kb *Cla*I DNA fragment containing *ctxB* was extracted from plasmid pRIT10810 and cloned in the unique *Cla*I site of pIC-20H. This recombinant plasmid provided a *Xba*I restriction fragment containing *ctxB*. In the second step, this *Xba*I fragment was cloned at the *Xba*I site of pMS1. The new plasmid, pMS2, contained an operon fusion between *yop51* and *ctxB*. B, Bam, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; X, Xba, *Xba*I; p, *yop51* promoter; Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Tc^R, tetracycline resistance.

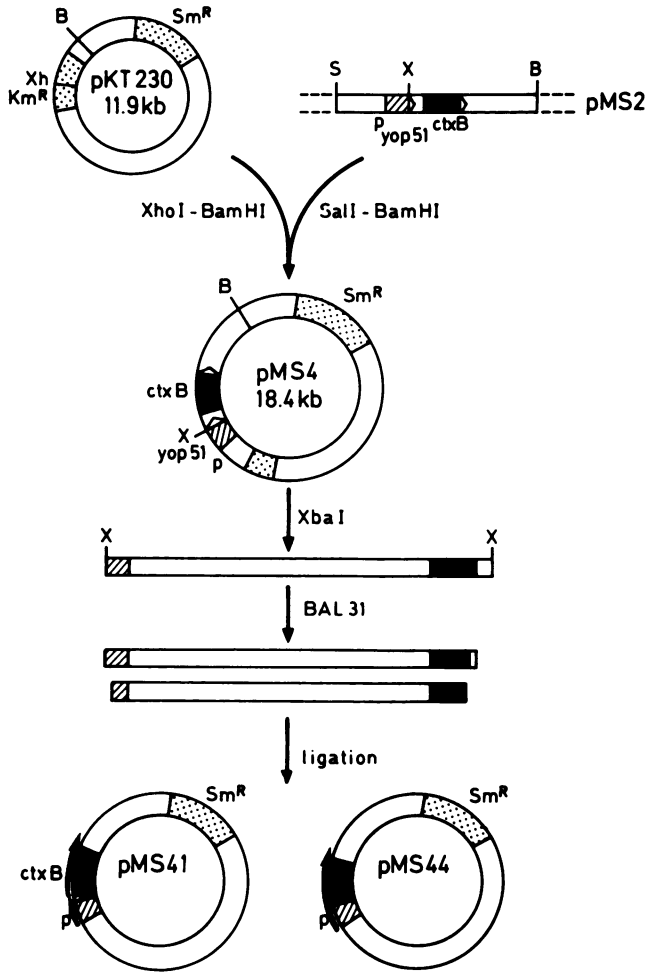

FIG. 2. Construction of pMS41 and pMS44. A 7.3-kb *Bam*HI-*Sal*I fragment containing the operon fusion *yop51-ctxB* was extracted from pMS2 and cloned in pKT230 digested by *Bam*HI and *Xho*I. The resulting plasmid, called pMS4, was linearized at *Xba*I and digested with BAL 31. Two types of plasmids were obtained. Some directed the production of free CT-B (e.g., pMS41). Others expressed a hybrid protein (e.g., pMS44). B, *Bam*HI; S, *Sal*I; X, *Xba*I; Xh, *Xho*I; p, promoter; Km^R, kanamycin resistance; Sm^R, streptomycin resistance.

deprived of Ca^{2+} showed that, like the Yops, CT-B only appeared at 37°C in absence of Ca^{2+} and not under the other conditions (data not shown). Analysis by SDS-polyacrylamide gel electrophoresis and immunoblotting showed that CT-B produced by *Y. enterocolitica* was still able to form a pentameric structure (data not shown). Since CT-B was produced as a free protein, we conclude that pMS41 carried an operon fusion between *ctxB* and *yop51'*. A Northern blot analysis (Fig. 4) confirmed that CT-B was translated from a hybrid *yop51'-ctxB* messenger. Sequencing of the junction *yop51'-ctxB* showed that the last 49 bp of *ctxA* remaining between *yop51'* and *ctxB* were in frame with *yop51'* and hence provided a stop codon to *yop51'* (data not shown).

Of the 20 recombinant plasmids, 4 (pMS44, pMS53, pMS65, and pMS72) directed the secretion of proteins with molecular masses between 26 and 29 kDa. Each construct produced a doublet of proteins of similar molecular mass, instead of a single protein. Both proteins reacted in immunoblots with an anti-CT-B serum as well as with an anti-Yops serum. Hence, we assumed that they were both hybrid Yop51-CT-B proteins. One of these recombinant plasmids, pMS44, encoded chimeric proteins of about 28 kDa (Fig. 3A). Sequencing of the junction *yop51'-ctxB* showed that the first 376 bp of *yop51* were joined in frame with the complete *ctxB* gene. Only the first nucleotide of *ctxB* was replaced by a nucleotide of *yop51* (data not shown).

To summarize, we constructed operon (pMS41) and gene (pMS44) fusions between *yop51* and *ctxB*. The operon fusion directed the synthesis of intracellular CT-B, while the gene fusion directed the secretion of hybrid proteins.

Plasma antibody response of mice inoculated with *Y. enterocolitica* containing pMS41. Strain W22703(pGC1256) was

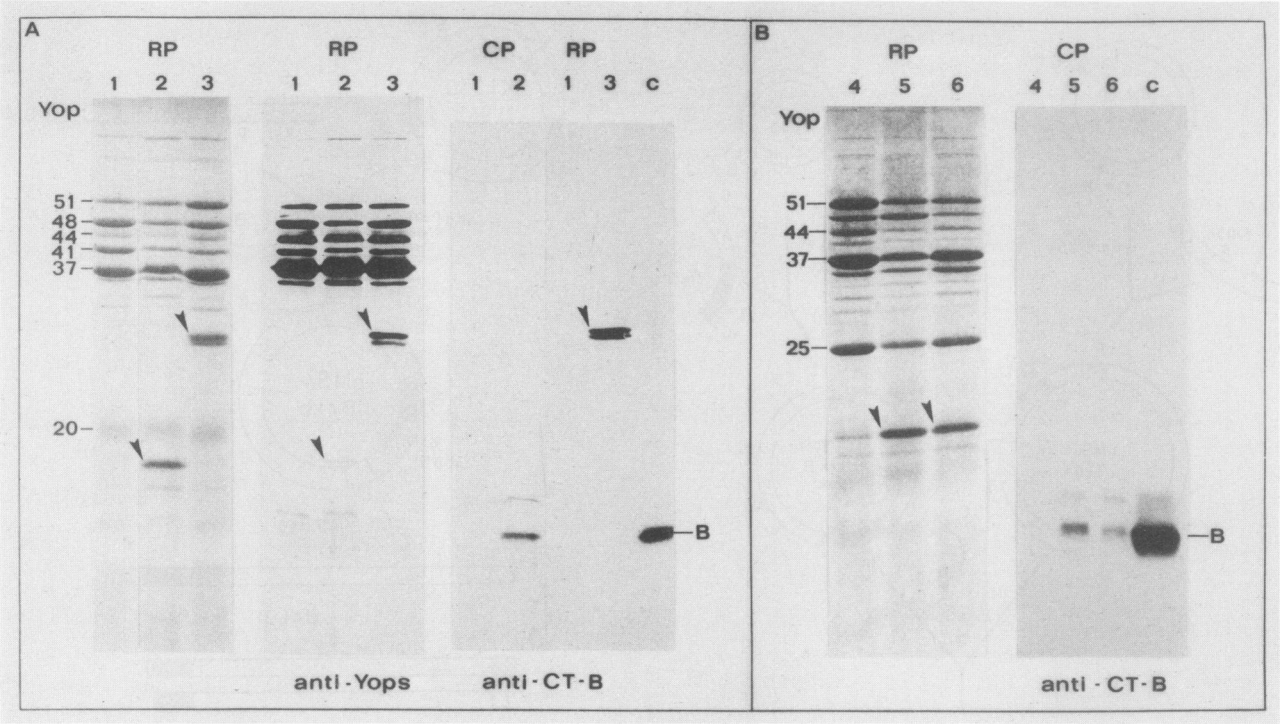

FIG. 3. Coomassie blue-stained SDS-polyacrylamide gel and immunoblots of released (RP) and cellular (CP) proteins of bacteria incubated at 37°C in the absence of Ca^{2+}. Immunoblots were revealed with a goat antiserum directed against CT-B or with a human antiserum directed against the Yops. (A) Lanes: 1, W22703(pGC1256); 2, W22703(pGC1256)(pMS41); 3, W22703(pGC1256)(pMS44); c, CT-B. (B) Lanes: 4, W22703(pBM7); 5, W22703(pBM7)(pMS41); 6, W22703(pBM7-4). Arrowheads indicate the truncated Yop51′ protein and the Yop51-CT-B chimeric protein. B, Monomeric form of CT-B. Molecular masses of Yops are indicated in kilodaltons to the left of each panel.

selected as a host strain for plasmid pMS41. Plasmid pGC1256 is a derivative of pYVe227 carrying a mini-Mu d*lac* element preventing the production of Yop25.

Strains W22703(pGC1256) and W22703(pGC1256)(pMS41) were given orally to BALB/c mice pretreated with the iron-chelating agent desferrioxamine B and iron dextran. Animals were inoculated at days 1 and 20 with live bacteria grown at RT. Sera were collected 15 days after the second inoculation and analyzed by immunoblotting. To visualize antibodies directed against the monomeric and the polymeric forms of the B subunit, we completely or partially denatured CT-B before loading it on SDS-polyacrylamide gels and electroblotting it. Antibodies directed against all the major Yops save Yop25 were detected in sera of the mice having received either strain. A response against CT-B appeared in sera of mice inoculated with the strain carrying pMS41. This response was only directed against the polymeric form of CT-B (Fig. 5).

To study the kinetics of total plasma IgG, we inoculated mice once (on day 1) with live bacteria grown at RT and bled them every week for 5 weeks. To have records every 3 days instead of every week, we divided the mice into two groups. The eight mice of the first group were bled for the first time on day 5, and the seven mice of the second group were bled for the first time on day 8. Plasma samples were tested by GM1-ELISA for their anti-CT-B IgG concentration. This concentration reached a maximum around day 20, decreased, and stabilized around day 30 (Fig. 6). Control experiments with mice inoculated with *Y. enterocolitica* W22703(pGC1256), i.e., a strain not producing CT-B, were negative (data not shown).

The antibody response induced by the strain W22703 (pGC1256)(pMS41) was also tested in C57BL/6 mice. Since these mice appeared to be more susceptible than BALB/c mice to oral infection by *Y. enterocolitica*, the mice were inoculated only once and they were not treated with iron and desferrioxamine B. The immunoblot analysis of the sera revealed again an antibody response against the polymeric form of the B subunit (data not shown).

Intestinal anti-CT-B IgA response of mice inoculated with *Y. enterocolitica* containing pMS41. In other experiments, we analyzed the intestinal secretions. The two *Yersinia* strains tested were W22703(pGC1256)(pMS41) and W22703(pBM7)(pMS41). Plasmid pBM7 is pYVe227 mutated in *yop20*. The BALB/c mice were inoculated exactly as for the previous experiments. Two batches of 15 mice were inoculated twice (on days 1 and 21). The intestinal secretions were collected on day 30 for IgA assays. After total IgA assay by ELISA, all the samples were diluted to normalize the total IgA concentrations at 1 $\mu g\ ml^{-1}$. The specific anti-CT-B IgA was then assayed by GM1-ELISA. The mouse intestinal secretions contained an anti-CT-B IgA activity (Fig. 7).

To ensure that the anti-CT-B IgA activity was due to secretory IgA, we normalized another sample of the intestinal secretions to 3 $\mu g\ ml^{-1}$ and assayed the secretory IgA directed against CT-B using an anti-secretory component serum. The response of mice inoculated with strain W22703(pBM7)(pMS41) was three times higher than the response obtained with W22703(pGC1256)(pMS41), but the response was significant in both cases (data not shown). We conclude that the anti-CT-B IgA detected in the secretions is indeed secretory IgA.

Integration of operon fusion *yop51-ctxB* in plasmid pBM7. The plasmid pMS41 was rather unstable in vivo. Three days after inoculation of three mice, only 39 ± 5% of the bacteria recovered from the distal ileum still contained pMS41. When

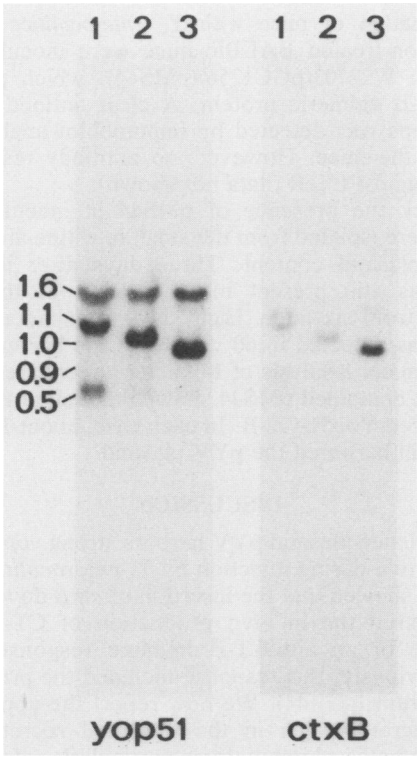

FIG. 4. Analysis of *yop51-ctxB* transcripts. RNAs prepared from bacteria incubated at 37°C in absence of Ca^{2+} were hybridized with a *yop51* (left) or *ctxB* (right) probe. Lanes 1, W22703(pGC1256)(pMS4); lanes 2, W22703(pGC1256)(pMS41); lanes 3, W22703(pGC1256)(pMS44). Sizes of mRNAs are indicated in kilobases. The 1.6-kb transcript is the *yop51* mRNA transcribed from pGC1256. The 0.5-kb transcript also derives from *yop51*, but it ends at a structure with a dyad symmetry occurring within *yop51* (34). This structure is only present in the *yop51* fragment of pMS4. The 1.1-, 1.0-, and 0.9-kb mRNAs are transcribed from the fusions on pMS4, pMS41, and pMS44, respectively.

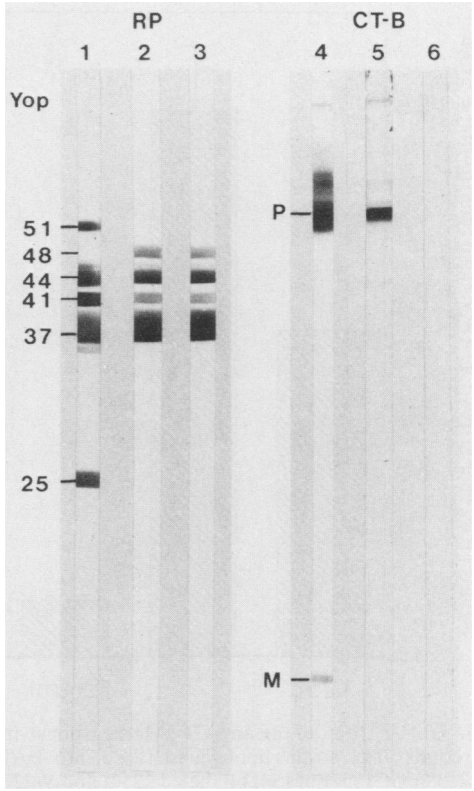

FIG. 5. Immunoblot analysis of BALB/c mouse sera. Serum samples were studied against the Yops prepared from *Y. enterocolitica* W22703(pYVe227) (RP) and against CT-B. Lanes: 1, rabbit anti-Yops serum; 2 and 5, pool of five sera, diluted 1/40 and 1/8, from mice infected with W22703(pGC1256)(pMS41); 3 and 6, pool of five sera, diluted 1/40 and 1/8, from mice infected with W22703(pGC 1256); 4, goat anti-CT-B serum. M and P indicate the monomeric and polymeric forms of CT-B, respectively. Molecular masses of Yops are indicated in kilodaltons.

the bacteria were collected 8 days after inoculation, only 17 ± 23% retained pMS41. In each case, 99 to 100% of bacteria still contained the pYV plasmid. To circumvent this problem of unstability, we integrated the operon fusion *yop51-ctxB* of pMS41 into the pYV plasmid itself. For this purpose, we selected pBM7, a pYVe227 derivative mutated in *yop20*.

A fragment of pMS41 containing the *yop51-ctxB* operon and a fragment containing the gentamicin resistance gene were first cloned together in the suicide plasmid pGP704. The recombinant plasmid, called pMSV14, was then transferred into *Y. enterocolitica* W22703(pBM7) where it could only be rescued by integration into pBM7. This occurred via a single crossover between the 1.5-kb fragment of pMSV14 containing the N-terminal part of *yop51* and the homologous region of pBM7 (Fig. 8).

The recombinant strain was analyzed by restriction and was called W22703(pBM7-4). Except for Yop20, this strain produced all the Yops including Yop51′ and CT-B.

To check the stability of pBM7-4, we isolated bacteria from the distal ileum of infected mice. Three days after inoculation, we observed that pMSV14 was still integrated in pBM7 in 99 ± 1% of the bacteria isolated from three mice. However, 8 days after inoculation, we only detected pMSV14 in 61 ± 48% of the bacteria isolated from four mice. In each case, all the bacteria still contained the pYV plasmid.

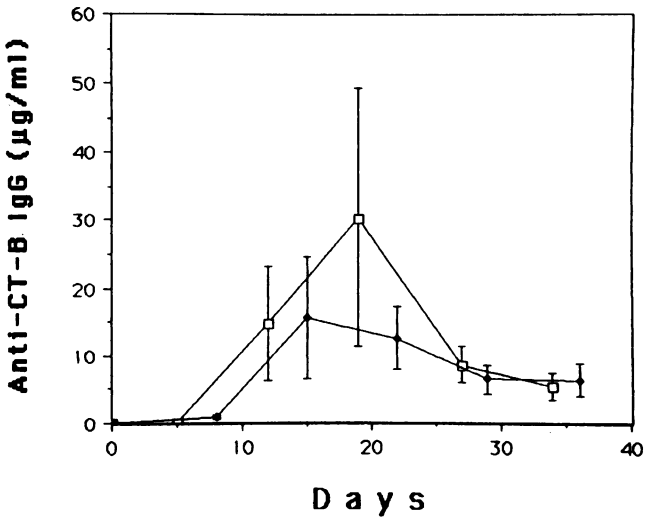

FIG. 6. GM1-ELISA of the plasma anti-CT-B IgG response. Fifteen BALB/c mice were orally inoculated once with strain *Y. enterocolitica* W22703(pGC1256)(pMS41). The mice, divided into two groups, were bled every week for 5 weeks. The eight mice of the first group (□) were bled for the first time on day 5, and the seven mice of the second group (◆) were bled for the first time on day 8. The anti CT-B IgG activity is given in micrograms per milliliter of plasma.

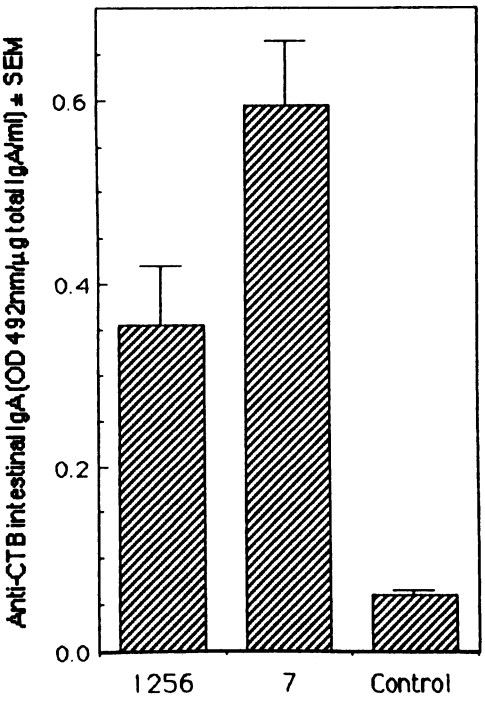

FIG. 7. GM1-ELISA of the anti-CT-B IgA response in the intestinal secretions. The strains inoculated twice into BALB/c mice were W22703(pGC1256)(pMS41) (labeled 1256) and W22703(pBM7) (pMS41) (labeled 7). The control consisted of intestinal secretions from untreated mice. The anti-CT-B IgA activity is expressed in OD_{492} units for 1 µg of total IgA ml^{-1}.

Immunization of mice with strain W22703(pBM7-4). Strain W22703(pBM7-4) was given to two groups of 5 and 15 C57BL/6 mice. Sera were collected 20 days after the single inoculation and analyzed by GM1-ELISA. A clear response against CT-B was observed. The serum titers varied from 140 to 1,490. A response was also detected by immunoblot against Yop25, Yop37, Yop44, and Yop48 (data not shown).

Immunization of mice with *Y. enterocolitica* containing pMS44. Iron-treated BALB/c mice were inoculated twice with strain W22703(pGC1256)(pMS44), which produces a Yop51-CT-B chimeric protein. A clear antibody response against Yops was detected by immunoblot analysis of the sera from the mice. However, no antibody response was detected against CT-B (data not shown).

To check the presence of pMS44 in inoculated mice, bacteria were isolated from the small intestine and analyzed for their plasmid content. Three days after inoculation, pMS44 was still present in 93 ± 11% of the bacteria recovered from six mice. Eight days after inoculation, the plasmid was detected in 60 ± 43% of the bacteria isolated from four mice. Analysis of 14 bacteria recovered from one mouse that contained pMS44 showed that all of the bacteria still produced Yop51-CT-B. In each case, about 100% of the bacteria still harbored the pYV plasmid.

DISCUSSION

The virulence plasmid pYV harbors strong *yop* promoters that are active during infection by *Y. enterocolitica*. In this report, we showed that the insertion of *ctxB* downstream of *yop51* ensured the in vivo production of CT-B and the appearance of an anti-CT-B immune response. As was shown previously, this response included the production of specific serum IgG (44). We now report the appearance of specific secretory IgA in the intestinal secretions. This finding of a mucosal immunity is particularly relevant in the context of vaccine development. It reinforces our previous findings (44) and suggests that *Y. enterocolitica* developed as a live carrier would probably give results as promising as those that have already been reported for *Salmonella* strains (6, 7, 42; for reviews, see references 16 and 19).

The antibody response against the B subunit after oral inoculation of mice was limited to the polymeric form of CT-B. This is in agreement with our immunoblot analysis of 10 serum samples from cholera-convalescent patients which showed that the antibody response was also directed only against the polymeric form of CT-B (data not shown). On the contrary, subcutaneous injection of mice with CT-B elicited

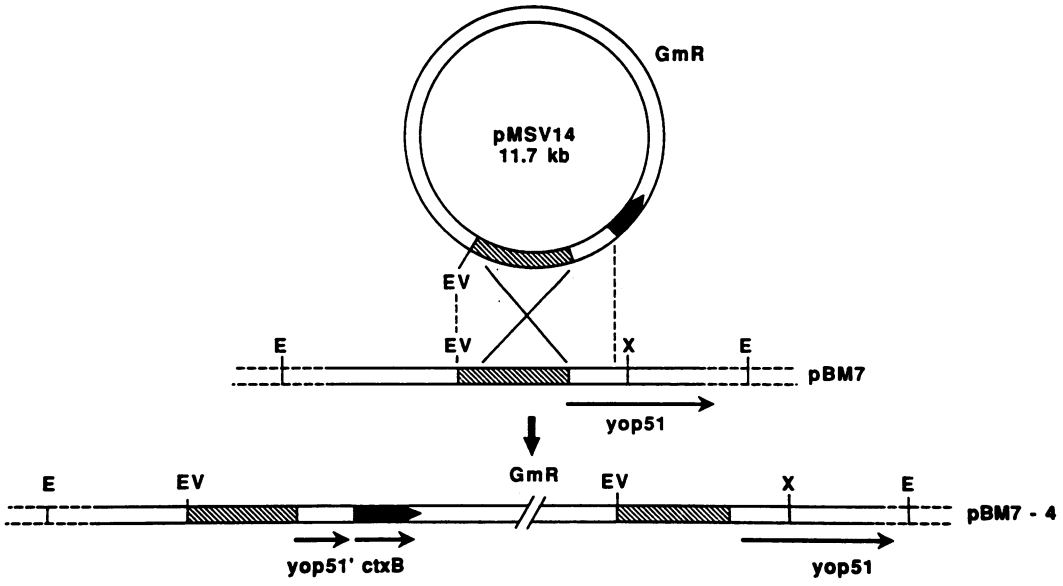

FIG. 8. Integration of pMSV14 in pBM7. Plasmid pMSV14 contains a 1.5-kb fragment homologous to pYV and hence to pBM7. E, *Eco*RI; EV, *Eco*RV; X, *Xba*I; GmR, gentamicin resistance.

antibodies directed against the monomeric form of CT-B (data not shown). The type of response thus seems to depend on the manner of inoculation.

The intracellular localization of CT-B in *Y. enterocolitica* was not unexpected since the cloning of *ctxB* in *E. coli* was also shown to lead to the production of a cell-bound CT-B (24, 40). The fact that we observed an antibody response against CT-B thus shows that the antigens do not have to be secreted outside the bacteria to stimulate the immune system. To test the immunogenicity of a foreign protein secreted by *Y. enterocolitica*, we constructed a strain that secreted a Yop51-CT-B hybrid protein. However, no antibody response was detected against CT-B after oral inoculation of mice with this strain. This cannot be due to the in vivo deletion of the hybrid gene because the plasmid encoding this gene was not easily lost during the infection and because all bacteria tested for Yop production after being recovered from mice were still able to produce Yop51-CT-B. Since the response induced by the strain producing free CT-B was only directed against the polymer, one can assume that the lack of answer reflects the inability of the chimeric protein to assemble into the characteristic B pentamer of CT-B. The presence of 125 amino acids derived from Yop51 at the amino-terminal end of CT-B could account for this inability to form the pentamer.

Two systems have been proposed to ensure the maintenance of a foreign gene in an attenuated *Salmonella* strain. The first one involves the use of an Asd^+ expression-cloning vector in a *Salmonella* strain lacking the *asd* gene (38). The second one is based on a recombination of the heterologous DNA in the chromosome (28). The pYV plasmid of *Y. enterocolitica* is very stable during the infection (2; this work). Hence, integration into pYV could also stabilize the foreign DNA introduced in the *Yersinia* carrier strain. This hypothesis was tested with our fusion *yop51-ctxB*. Despite the fact that the number of copies of the recombinant *yop51-ctxB* operon was lower in the integrated pYV construct than in the RSF1010 construct, the former also elicited a clear antibody response. However, the integration was constructed by a single crossover, and a second homologous recombination could lead to the excision and loss of the recombinant operon. Despite the fact that this event was found to occur in vivo, the stability of this construction was better than that based on RSF1010. The exact calculation of this stability is, however, tedious since it cannot be done in vitro because the induction of the *yop* regulon correlates with a severe growth restriction favoring the emergence of cured bacteria. Moreover, the bacteria should be recovered from many different mice since all the bacteria collected from one animal could derive from a small number of cells that initiated the infection. This was not done because our construct is clearly not the optimal one. However, the purpose of this work was not to make a real vaccinal strain but rather to demonstrate the potentiality of the system.

Finally, the use of *Y. enterocolitica* as a live carrier for protective antigens needs to ensure the safety of the strain. In *Salmonella* species, several mutations such as *aro* (27), Δ*cya* Δ*crp* (15), *ompR* (18), and *phoP* (23) have been proposed to serve this purpose. *aroA* mutants of *Y. enterocolitica* from serotype O:8 have recently been constructed (5). However, this mutant does not persist longer than 5 days in mice and three doses of 10^9 to 10^{10} bacteria are required to induce a protection against *Y. enterocolitica*. According to the authors, these mutants are less protective than the *aroA Salmonella* strains. Hence, they could probably not be used as live carriers. An ideal way to reduce the virulence of a live *Yersinia* vaccinal strain could be the inactivation of one or several *yop* genes. Complete safety of the carrier would also require the deletion of the gene encoding the enterotoxin Yst of *Y. enterocolitica*. This gene has now been cloned and sequenced (Delor and Cornelis, submitted), which will permit construction of adequate mutants.

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LITERATURE CITED

1. **Attwood, S. E. A., M. T. Cafferkey, and F. B. V. Keane.** 1989. *Yersinia* infections in surgical practice. Br. J. Surg. **76**:499–504.
2. **Bakour, R., G. Balligand, Y. Laroche, G. Cornelis, and G. Wauters.** 1985. A simple adult mouse test for tissue invasiveness in *Yersinia enterocolitica* strains of low experimental virulence. J. Med. Microbiol. **19**:237–246.
3. **Biot, T., and G. Cornelis.** 1988. The replication, partition and *yop* regulation of the pYV plasmids are highly conserved in *Yersinia enterocolitica* and *Y. pseudotuberculosis*. J. Gen. Microbiol. **134**:1525–1534.
4. **Bölin, I., and H. Wolf Watz.** 1988. The plasmid-encoded Yop2b protein of *Yersinia pseudotuberculosis* is a virulence determinant regulated by calcium and temperature at the level of transcription. Mol. Microbiol. **2**:237–245.
5. **Bowe, F., P. O'Gaora, D. Maskell, M. Cafferkey, and G. Dougan.** 1989. Virulence, persistence, and immunogenicity of *Yersinia enterocolitica* O:8 *aroA* mutants. Infect. Immun. **57**: 3234–3236.
6. **Brown, A., C. E. Hormaeche, R. Demarco de Hormaeche, M. Winther, G. Dougan, D. J. Maskell, and B. A. D. Stocker.** 1987. An attenuated *aroA Salmonella typhimurium* vaccine elicits humoral and cellular immunity to cloned β-galactosidase in mice. J. Infect. Dis. **155**:86–92.
7. **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.
8. **Cornelis, G., T. Biot, C. Lambert de Rouvroit, T. Michiels, B. Mulder, C. Sluiters, M.-P. Sory, M. Van Bouchaute, and J.-C. Vanooteghem.** 1989. The *Yersinia yop* regulon. Mol. Microbiol. **3**:1455–1459.
9. **Cornelis, G., and C. Colson.** 1975. Restriction of DNA in *Yersinia enterocolitica* detected by recipient ability for a derepressed R factor from *Escherichia coli*. J. Gen. Microbiol. **87**:285–291.
10. **Cornelis, G., Y. Laroche, G. Balligand, M.-P. Sory, and G. Wauters.** 1987. *Yersinia enterocolitica*, a primary model for bacterial invasiveness. Rev. Infect. Dis. **9**:64–87.
11. **Cornelis, G., C. Sluiters, C. Lambert de Rouvroit, and T. Michiels.** 1989. Homology between VirF, the transcriptional activator of the *Yersinia* virulence regulon, and AraC, the *Escherichia coli* arabinose operon regulator. J. Bacteriol. **171**: 254–262.
12. **Cornelis, G., M.-P. Sory, Y. Laroche, and I. Derclaye.** 1986. Genetic analysis of the plasmid region controlling virulence in *Yersinia enterocolitica* O:9 by mini-Mu insertions and *lac* gene fusions. Microb. Pathogen. **1**:349–359.
13. **Cornelis, G., J. C. Vanooteghem, and C. Sluiters.** 1987. Transcription of the *yop* regulon from *Y. enterocolitica* requires trans acting pYV and chromosomal genes. Microb. Pathogen. **2**: 367–379.
14. **Cover, T. L., and R. C. Aber.** 1989. *Yersinia enterocolitica*. N.

Engl. J. Med. **6**:16–24.
15. **Curtiss, R., III, and S. M. Kelly.** 1987. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. **55**:3035–3043.
16. **Curtiss, R., III, S. M. Kelly, P. A. Gulig, and K. Nakayama.** 1989. Selective delivery of antigens by recombinant bacteria. Curr. Top. Microbiol. Immunol. **146**:35–49.
17. **Derbyshire, K. M., G. Hatfull, and N. Willets.** 1987. Mobilization of the non-conjugative plasmid RSF1010: a genetic and DNA sequence analysis of the mobilization region. Mol. Gen. Genet. **206**:161–168.
18. **Dorman, C. J., S. Chatfield, C. F. Higgins, C. Hayward, and G. Dougan.** 1989. Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: *ompR* mutants are attenuated in vivo. Infect. Immun. **57**:2136–2140.
19. **Dougan, G.** 1989. Molecular characterization of bacterial virulence factors and the consequences for vaccine design. The 1988 Fleming Lecture. J. Gen. Microbiol. **135**:1397–1406.
20. **Elson, O. C., W. Ealding, and J. Lefkowitz.** 1984. A lavage technique allowing repeated measurement of IgA antibody in mouse intestinal secretions. J. Immunol. Methods **67**:101–108.
21. **Finkelstein, R. A.** 1984. Cholera, p. 107–136. *In* R. Germanier (ed.), Bacterial vaccines. Academic Press, Inc. (London) Ltd., London.
22. **Forsberg, A., and H. Wolf Watz.** 1988. The virulence protein Yop5 of *Yersinia pseudotuberculosis* is regulated at the transcriptional level by plasmid-pIB1-encoded trans-acting elements controlled by temperature and calcium. Mol. Microbiol. **2**: 121–133.
23. **Galan, J. E., and R. Curtiss III.** 1989. Virulence and vaccine potential of *phoP* mutants of *Salmonella typhimurium*. Microb. Pathogen. **6**:433–443.
24. **Gennaro, M. L., P. J. Greenaway, and D. A. Broadbent.** 1982. The expression of biologically active cholera toxin in *Escherichia coli*. Nucleic Acids Res. **10**:4883–4890.
25. **Goetzl, J., and H. Metzger.** 1970. Affinity labeling of a mouse myeloma protein which binds nitrophenyl ligands. Kinetics of labeling and isolation of a labeled peptide. Biochemistry **9**: 1267–1278.
26. **Heesemann, J., B. Algermissen, and R. Laufs.** 1984. Genetically manipulated virulence of *Yersinia enterocolitica*. Infect. Immun. **46**:105–110.
27. **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.
28. **Hone, D., S. Attridge, L. van den Bosch, and J. Hackett.** 1988. A chromosomal integration system for stabilization of heterologous genes in *Salmonella* based vaccine strain. Microb. Pathogen. **5**:407–418.
29. **Isberg, R. R.** 1989. Mammalian cell adhesion functions and cellular penetration of enteropathogenic *Yersinia* species. Mol. Microbiol. **3**:1449–1453.
30. **Lemaître-Coelho, I., C. André, and J.-P. Vaerman.** 1977. Murine secretory component. Protides Biol. Fluids Proc. Colloq. **25**: 891–894.
31. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Marsh, J. L., M. Erfle, and E. J. Wykes.** 1984. The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene **32**:481–485.
33. **Mekalanos, J. J., D. J. Swartz, G. D. N. Pearson, N. Harford, F. Groyne, and M. de Wilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature (London) **306**:551–557.
34. **Michiels, T., and G. Cornelis.** 1988. Nucleotide sequence and transcription analysis of *yop51* from *Yersinia enterocolitica* W22703. Microb. Pathogen. **5**:449–459.
35. **Miller, V. L., and S. Falkow.** 1988. Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. Infect. Immun. **56**:1242–1248.
36. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170**:2575–2583.
37. **Mulder, B., T. Michiels, M.-P. Sory, M. Simonet, and G. Cornelis.** 1989. Identification of additional virulence determinants on the pYV plasmid of *Y. enterocolitica* W227. Infect. Immun. **57**:2534–2541.
38. **Nakayama, K., S. M. Kelly, and R. Curtiss III.** 1988. Construction of an Asd+ expression-cloning vector: stable maintainance and high level expression of cloned genes in a *Salmonella* vaccine strain. Bio/Technology **6**:693–697.
39. **Nugent, M. E., D. H. Bone, and N. Datta.** 1979. A transposon, Tn*732*, encoding gentamicin/tobramycin resistance. Nature (London) **282**:422–423.
40. **Pearson, G. D. N., and J. J. Mekalanos.** 1982. Molecular cloning of *Vibrio cholerae* enterotoxin genes in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **79**:2976–2980.
41. **Pierre, P., A. Langendries, and J.-P. Vaerman.** 1988. Cholera toxin neutralization: comparison of purified serum IgG and biliary secretory IgA antibody. Immunol. Lett. **18**:51–56.
42. **Poirier, T. P., M. A. Kehoe, and E. H. Beachey.** 1988. Protective immunity evoked by oral administration of attenuated *aroA Salmonella typhimurium* expressing cloned streptococcal M protein. J. Exp. Med. **168**:25–32.
43. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–791.
44. **Sory, M.-P., and G. Cornelis.** 1988. *Yersinia enterocolitica* O:9 as a potential live oral carrier for protective antigens. Microb. Pathogen. **4**:431–442.
45. **Svennerholm, A.-M., and J. Holmgren.** 1978. Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM1-ELISA) procedure. Curr. Microbiol. **1**:19–23.
46. **Takao, T., N. Tominaga, S. Yoshimura, Y. Shimonishi, S. Hara, T. Inoue, and A. Miyama.** 1985. Isolation, primary structure and synthesis of heat-stable enterotoxin produced by *Yersinia enterocolitica*. Eur. J. Biochem. **152**:199–206.
47. **Wormser, G. P., and G. T. Keusch.** 1981. *Yersinia enterocolitica*: clinical observations, p. 83–93. *In* E. J. Bottone (ed.), *Yersinia enterocolitica*. CRC Press, Boca Raton, Fla.