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Development of *Shigella sonnei* Live Oral Vaccines Based on Defined rfb_{Inaba} Deletion Mutants of *Vibrio cholerae* Expressing the *Shigella* Serotype D O Polysaccharide

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Previous experimentation has highlighted a number of difficulties in the development of carrier-based bivalent vaccines (J.-F. Viret and D. Favre, Biologicals 22:361–372, 1994). In an attempt to obviate these problems, we decided to express the *Shigella sonnei* serotype D O polysaccharide (O-PS) in rough *Vibrio cholerae* carrier strains. Toward this aim, a series of defined rfb_{Inaba} deletion ($\Delta rfb_{\text{Inaba}}$) mutants of the cholera vaccine strain *V. cholerae* CVD103-HgR (O1 Inaba serotype) and derivatives bearing the chromosomally integrated locus encoding the *S. sonnei* O-PS were constructed and characterized. The various mutations disrupt genes thought to be involved in either the synthesis of perosamine, the synthesis of 3-deoxy-L-glycero tetronic acid, or the O-PS transport functions together with synthesis of the perosamine synthetase. Some deletions were obtained only in strains expressing the heterologous lipopolysaccharide (LPS). Viable $\Delta rfb_{\text{Inaba}}$ deletions in CVD103-HgR profoundly altered some of its phenotypic properties. The same deletions present in CVD103-HgR derivatives expressing the heterologous LPS affected their phenotypes only to a lesser extent. Only in strains in which perosamine synthesis was specifically abolished could high amounts of core-bound *S. sonnei* O-PS be synthesized. Two such strains (CH21, which expresses both the R1 core and the *S. sonnei* O-PS, and CH22, which expresses only the latter antigenic determinant) were further analyzed and were found to be indistinguishable from CVD103-HgR with regard to lack of enterotoxin activity, choleragenoid production, mercury resistance, pilin production, and, for CH22, motility. Mice immunized with CH22 produced high titers of *S. sonnei* O-PS-specific antibodies.

Enteric diseases account for an estimated 25% of all infant deaths in developing countries in addition to significant morbidity and mortality among both resident adults and travellers (3). A number of bacterial pathogens are leading causes of such illnesses. Treatment often consists of a combination of rehydration and antibiotic therapy. However, the latter can be greatly complicated by the increasing prevalence of resistant strains. Prevention of such diseases through the use of vaccination campaigns will, therefore, become of greater importance in the future. However, effective vaccines currently exist only against *Salmonella typhi* and O1 strains of *Vibrio cholerae* (22, 39, 40). The development of new and improved vaccines against enteric pathogens has been given a high priority by international agencies (68).

Shigella spp. can cause disease in both developed and developing countries. Symptoms range from diarrhea to bacillary dysentery depending on the infecting organism. Disease is caused by the invasion of the colonic mucosae and multiplication within enterocytes concomitant with cell-to-cell spread. The development of mucosal lesions by this process accounts for the mucus often seen in the stools of patients suffering from a *Shigella* infection. Prior infection can confer a high degree of immunity on subsequent infection with the homologous species (42). This has served as the impetus to develop vaccines which can induce an immune response similar to that observed after natural infection.

Natural immunity to infection appears to be mediated by anti-lipopolysaccharide (LPS) antibodies directed against the O polysaccharide (O-PS) moiety (12, 13, 27). This has led to the construction of a variety of vaccines containing O-PS, including conjugates for parenteral administration (63), proteosomes (50), *Shigella* sp. live attenuated vaccine candidates (17, 32, 41, 49), and recombinant heterologous bacterial carrier strains (6, 10, 18, 25, 65). While expression of *Shigella sonnei* O-PS in the live oral *S. typhi* Ty21a vaccine strain gave initially promising results (4), genetic instability of the construct precluded its use as a vaccine (26).

The O-PS is a linear polymer generally composed of repeating blocks of sugar residues. It is proximally linked to the LPS core region which is attached to the lipid A moiety embedded into the outer leaflet of the outer membrane (43). LPS core biosynthesis is directed by genes contained in the *rfa* locus, which is chromosomally located. Seven distinct core types are currently recognized within the family *Enterobacteriaceae* and are termed Ra, R1, R2, R3, R4, K-12, and B (29, 43). *S. sonnei* possesses the R1 type core. The chemical structures of the *V. cholerae* LPS core vary considerably with respect to those of the *Enterobacteriaceae* (37). The O-PS of *V. cholerae* O1, which determines the Inaba/Ogawa O serotypes, is composed of 17 to 18 perosamine residues, each of which is acylated with 3-deoxy-L-glycero-tetronic acid. D-Quinovosamine has also been found at a low ratio; however, its location within the O-PS molecule remains unclear (28, 35, 53). The O-PS of *S. sonnei* is composed of a disaccharide, 2-amino-2-deoxy-L-altruronic acid and 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose (5, 34). The *rfb* locus, which encodes the O-PS moiety, is chromosomally located for *V. cholerae*, whereas it is plasmid borne in *S. sonnei* (46, 58). In *S. sonnei*, an *rfb*-linked gene, termed *rfc*, codes for an O-antigen polymerase which is believed to be responsible for assembling the disaccharide units into long-chain repeats. Both the entire rfb/rfc_{sonnei} and rfa_{R1} loci have been cloned and characterized elsewhere (66).

Our laboratory has focused on the expression of foreign LPS

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TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype and/or description[a]	Source or reference
E. coli		
HB101	*supE44 ara-14 galK2 lacY1 proA2 rpsL20 xyl-5 mtl-1 recA13* Δ(*mcrC-mrr*)	56
DH5α	F^- Φ*80dlacZ*Δ*M15* Δ(*lacZYA-argF*) *U169 deo recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ^- *thi-1 gyrA96 relA1 phoA*	56
S17.1	*thi-1 pro hsdR* Tp^r Sm^r RP4-2[Tc::Mu(Km::Tn*7*)]	60
S. sonnei		
482-79(pWR105)	Phase I (smooth LPS)	5
V. cholerae		
CVD103-HgR	Δ*ctxA hlyA*::*mer* (Hg^r)	36
CH3	Δ*ctxA hlyA*::*mer hlyA*::*rfb*/rfc_{sonnei}	65, 67
CH9	Δ*ctxA hlyA*::*mer hlyA*::*rfb*/rfc_{sonnei} *hlyB*::rfa_{R1}	65, 67
CH13	CH3 Δ*rfbDEGHI*	This work
CH14	CH9 Δ*rfbDEGHI*	This work
CH15	CVD103-HgR Δ*rfbN*	This work
CH17	CH9 Δ*rfbN*	This work
CH19	CVD103-HgR Δ*rfbAB*	This work
CH21	CH9 Δ*rfbAB*	This work
CH22	CH19 *hlyA*::*rfb*/rfc_{sonnei}	This work
Plasmids		
pLAFR5	Broad-host-range cosmid vector (21.5 kb)	33
pMTL22p	High-copy-number plasmid vector	7
pMAK700oriT	Mobilizable thermosensitive suicide vector used for chromosomal deletions and gene integration	65
pSSVI255-3	rfb_{Inaba} locus cloned into pLAFR5	This work
pSSVI255-5	rfb_{Inaba} locus cloned into pLAFR5	This work
pSSVI255-7	rfb_{Inaba} locus cloned into pLAFR5	This work
pSSVI205-1	pMAK700oriT carrying the entire insert of pSSVI255-7 from which the three internal *Sac*I fragments were deleted. The insert contains also ca. 1 kb of pLAFR5 DNA	This work
pSSVI205-2	Same as pSSVI205-1 but inserted in opposite orientation	This work
pSSVI255-12	pMAK700oriT carrying the *Hin*dIII-*Sal*I fragment of pSSVI255-7 at coordinates 8420 to 17730, from which the central *Bam*HI fragment was deleted	This work
pSSVI255-19	pMAK700oriT carrying the *Cla*I fragment from pSSVI255-7 at coordinates 15770 to 21030, from which the central *Sal*I fragment was deleted	This work
pSSVI255-20	pMAK700oriT carrying the *Sac*I-*Bam*HI fragment from pSSVI255-7 at coordinates 5000 to 10340 from which the central *Hin*dIII fragment was deleted	This work
pSSVI201-1	Integration plasmid for the *rfb*/rfc_{sonnei} locus	65

[a] Coordinates correspond to those given in Fig. 2.

antigens in the live attenuated cholera vaccine strain CVD103-HgR (31, 36). This strain was constructed by deleting 94% of the toxic, enzymatically active subunit A of cholera toxin from strain *V. cholerae* 569B. Furthermore, a mercury resistance locus (*mer* genes) was inserted into the nonessential *hlyA* gene to provide a readily identifiable marker. CVD103-HgR possesses an excellent safety profile in children and adults. This, together with the fact that selective adherence of *V. cholerae* to M cells in the gut for antigen sampling may provide for a strong stimulation of the common mucosal immune system (11, 51), makes of CVD103-HgR an excellent candidate carrier strain. However, previous attempts by us and others have shown that coexpression of two O-PS molecules can have undesirable consequences such as low yield and/or masking of the heterologous O-PS (19, 65, 66). Therefore, an optimal approach may be to express only the heterologous O-PS in the carrier strain.

In the present study, Δrfb_{Inaba} mutants of CVD103-HgR and derivatives thereof bearing the chromosomally recombined *rfb*/rfc_{sonnei} locus alone or together with the rfa_{R1} locus have been constructed in an attempt to develop vaccine strains capable of expressing high levels of *S. sonnei* O-PS covalently coupled to the LPS core. Various constructs were genetically and physiologically characterized. One strain which appeared to possess the greatest potential for vaccine use was tested for its ability to induce anti-*S. sonnei* immune response.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used are listed in Table 1. The growth medium for liquid and solid cultures of *Escherichia coli* was Luria-Bertani broth (LB) (47). For *V. cholerae*, either LB broth, brain heart infusion broth (BHI), or colonization factor broth (14) was used. Plasmid-containing cells were selected on media supplemented with the following concentrations of antibiotics: ampicillin, 100 μg/ml; spectinomycin, 50 μg/ml; tetracycline, 12.5 μg/ml; and chloramphenicol, 17 μg/ml.

Molecular cloning techniques. Standard cloning techniques and Southern hybridization were according to published methods (56). Chromosomal DNA isolation was by the method described by Pitcher et al. (52). DNA fragment isolation was according to the method described by Favre (15). Both nonradioactive probe labelling with digoxigenin-1-dUTP and detection of hybrid DNA made use of a commercial kit (Digoxigenin labelling and detection kit; Boehringer Mannheim AG, Rotkreuz, Switzerland) and were performed according to the manufacturer's specifications. For the *V. cholerae* gene bank, chromosomal DNA fragments were generated by partial *Sau*3A digestion. DNA fragments were size fractionated on a sucrose gradient. Fractions containing 20- to 30-kb fragments were purified and ligated to *Bam*HI-*Sca*I-cut cosmid pLAFR5. The ligated mixture was packaged in vitro (Gigapack II Plus packaging kit; Stratagene GMBH, Zürich, Switzerland). The packaged cosmids were then transfected into *E. coli* HB101, and the resulting culture was plated onto tetracycline-containing LB plates to select for transfectants.

Screening of rfb_{Inaba} clones. Colonies representative of a complete *V. cholerae* gene bank were blotted onto nitrocellulose filters. The filters were air dried and blocked at room temperature for 10 min with phosphate-buffered saline (PBS [pH 7.4]) containing 10% (vol/vol) horse serum. The filters were then washed three times with PBS and incubated at room temperature for 30 to 90 min with the anti-cholera O-PS monoclonal antibody VCO4 (24). The filters were again washed three times with PBS and incubated for 30 min in PBS containing 5%

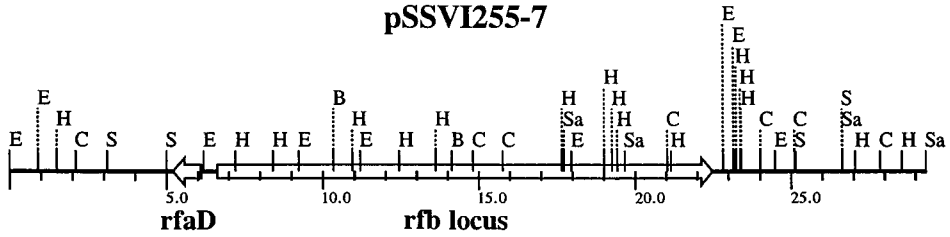

FIG. 1. Restriction map of cosmid pSSVI255-7 (insert only) carrying the rfb_{Inaba} region. Arrows point to the directions of transcription of the indicated loci. Scale below the line is in kilobases. Restriction enzymes: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I; Sa, *Sal*I. Recognition sites for the restriction enzymes located between the internal *Sac*I sites (coordinates, ca. 5.0 to 25.1) are inferred both from sequencing data (44) and our current findings.

(vol/vol) horse serum and a 1/1,000 dilution of peroxidase-conjugated goat anti-mouse immunoglobulin M antibodies (Tago, Burlingame, Calif.). The filters were washed as described above and were incubated with detection solution (50 mM Tris-HCl [pH 7.4] and 15 mM NaCl containing 0.05% [wt/vol] chlornaphthol and 0.1% [vol/vol] H_2O_2) until color developed. The reaction was stopped by washing with H_2O.

Plasmid transfer. Plasmid transfer between *E. coli* strains was by electroporation at 2.5 kV, 200 Ω, and 25 μF (Gene Pulser; Bio-Rad Laboratories AG, Glattbrugg, Switzerland). Transfer from *E. coli* to *V. cholerae* was by conjugation on solid medium with the RP4/RK2 transfer system carried in *E. coli* S17.1 (60). Briefly, donor and recipient were mixed at an estimated cell ratio of 1:50 to 1:100 onto an LB plate, and the plate was incubated overnight at 30°C. The mixture was then streaked out onto a number of selective LB plates, which were incubated at 30°C. Despite the unavailability of easy counterselection of the donor strain, transconjugants could be detected on the basis of colony morphology. Typical *E. coli* colonies are opaque, whereas *V. cholerae* colonies are translucent.

Chromosomal integration of rfb_{sonnei} genes and generation of rfb_{Inaba} deletions. The procedure leading to chromosomal integration of pMAK700oriT-derived vectors has been described in detail (65). Briefly, deletion plasmids were propagated in *V. cholerae* cells by incubation at 30°C on LB chloramphenicol plates. Selection for cells harboring chromosomally integrated plasmids was accomplished by growth on LB chloramphenicol plates at 41 to 42°C. Pools of single colonies were propagated by two or three rounds of subculturing on new preheated (42°C) LB chloramphenicol plates. Finally, pools of colonies were resuspended in liquid LB medium, and suitable dilutions were spread onto LB plates incubated at 30°C. Arising colonies were screened for chloramphenicol sensitivity before any further subculturing. Chloramphenicol-sensitive colonies were then immunoscreened for the desired phenotype, either expression of *S. sonnei* O-PS or loss of expression of *V. cholerae* O-PS.

LPS preparations. For LPS small scale preparations (minipreps), 1 ml of an overnight *V. cholerae* culture grown in BHI medium at 30°C was pelleted and resuspended in 1 ml of 0.85% NaCl. The cell suspension was again pelleted and the cells were lysed in 150 μl of LPS buffer (100 mM Tris-HCl [pH 6.8], 10% [wt/vol] glucose, 2% [wt/vol] sodium dodecyl sulfate [SDS], 4% [vol/vol] 2-mercaptoethanol, 0.001% [wt/vol] bromophenol blue). The lysate was boiled for 10 min, cooled on ice, and further incubated for 1 to 2 h at 55°C in the presence of 30 μl of a 3.5-mg/ml proteinase K solution in LPS buffer. A 10- to 15-μl volume of the LPS miniprep were then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The large-scale purification of LPS used as coating antigens for the enzyme-linked immunosorbent assays (ELISA) and for SDS-PAGE analysis was by the hot phenol-water technique as previously described (9).

SDS-PAGE and Western blots (immunoblots) of LPS minipreps. PAGE, Western blotting, and detection of LPS with specific anti-LPS monoclonal antibodies (MAbs) have been described elsewhere (66). Detection of *S. sonnei* and *V. cholerae* O-PS was with MAbs Sh5S (64) and VCO4 (24), respectively.

Physiological characterizations. Cell morphology, aggregation, filamentation, and motility were determined by phase-contrast microscopy. For determination of pilin synthesis, cultures were grown at 30°C overnight in colonization factor broth medium (pH 6.5) with slow shaking. Whole-cell lysates were then analyzed by SDS-PAGE and Western blotting with a TcpA-specific rabbit polyclonal antibody (kindly provided by J. B. Kaper, Center for Vaccine Development, Baltimore, Md.). The second antibody was peroxidase-conjugated goat anti-rabbit immunoglobulin G (H+L) (Bio-Rad Laboratories AG).

Tests for mercury resistance, Y1-adrenal cell activity, and GM1-ELISA binding activity were performed as described elsewhere (16, 55, 61).

Genetic stability. A culture of the test strain was repeatedly grown to stationary phase at 37°C in LB medium, diluted 200-fold in the same medium, and further incubated to stationary phase at 37°C. At each round, dilutions of the stationary culture were plated on LB medium for determination of stability. Genetic stability was defined as the proportion of colonies still expressing the desired phenotype (expression of *S. sonnei* O-PS or loss of *V. cholerae* O-PS) after 50 or more generations of growth.

Immunizations. Bacterial cells used for immunization were heat inactivated (60 min, 65°C), pelleted, and resuspended in 0.85% NaCl at a concentration of 5×10^8 cells per ml. Suitable aliquots were kept frozen for subsequent immunization. Groups of seven mice (18- to 20-g female IV:NMR1 mice) were immunized intramuscularly at days 0 and 14 with 5×10^7 cells (0.1 ml) and then given booster injections intraperitoneally at day 21 with 10^8 cells (0.2 ml). An additional group of seven mice were not immunized (negative control). All mice were sacrificed on day 28, and their blood was collected and processed for serum. Sera were stored at −20°C.

Determination of anti-LPS antibodies. The sera from mice were individually tested for the presence of LPS-specific antibodies with purified *S. sonnei* phase 1 or *V. cholerae* Inaba LPS as immobilized antigens in an ELISA as previously described (64).

RESULTS

Cloning of the rfb_{Inaba} locus from CVD103-HgR. A *Sau*3A chromosomal gene bank of *V. cholerae* vaccine strain CVD103-HgR was constructed in cosmid vector pLAFR5 and established in *E. coli* HB101. A number of clones containing the entire rfb_{Inaba} locus were isolated following colony immunoblot screening with the VCO4 MAb. Rough maps were constructed for three overlapping cosmid clones, pSSVI255-3, pSSVI255-5, and pSSVI255-7. Comparison of these maps with that of an El Tor Ogawa strain inferred from its nucleotide sequence (44, 45) suggests a high degree of overall conservation at the *rfb* locus. Cosmid pSSVI255-7 (Fig. 1) was chosen for subsequent studies.

Construction of CVD103-HgR derivatives with Δ*rfb* deletions. (i) Rationale for the construction of deletion mutagenesis vectors. Recent knowledge about the genetic organization of the rfb_{Ogawa} locus and the predicted functions of the encoded genes (44, 45) enabled us to target rfb_{Inaba} deletions to specific functions and to implement them into the chromosomes of selected *V. cholerae* strains. First, defined fragments of cosmid pSSVI255-7 were subcloned into plasmid vectors. Second, inner fragments from the inserts of the latter plasmids were excised so that adjacent sequences would be able to recombine with, and ultimately replace, homologous chromosomal regions in suitable hosts. Third, the new inserts were transferred into the suicide vector pMAK700oriT, giving rise to a series of deletion vectors. Finally, these deletion vectors were mobilized into strain CVD103-HgR, CH3, or CH9, in which chromosomal *rfb* deletions were desired. Chromosomal integration was achieved as outlined in Materials and Methods. Screening for Cm^s and then immunoscreening of rfb_{Inaba} expression with MAb VCO4 allowed for the isolation of colonies issued from cells that had lost the wild-type sequence and maintained the mutated locus. Various deletion mutants of interest are described below.

(ii) Construction of deletion mutagenesis vectors and *V. cholerae* deletion mutants. Figure 2 summarizes the various deletion vectors that were generated. Plasmids pSSVI205-1 and pSSVI205-2 were constructed by removal of 23.5 kb of DNA between the outer *Sac*I sites in pSSVI255-7 and subclon-

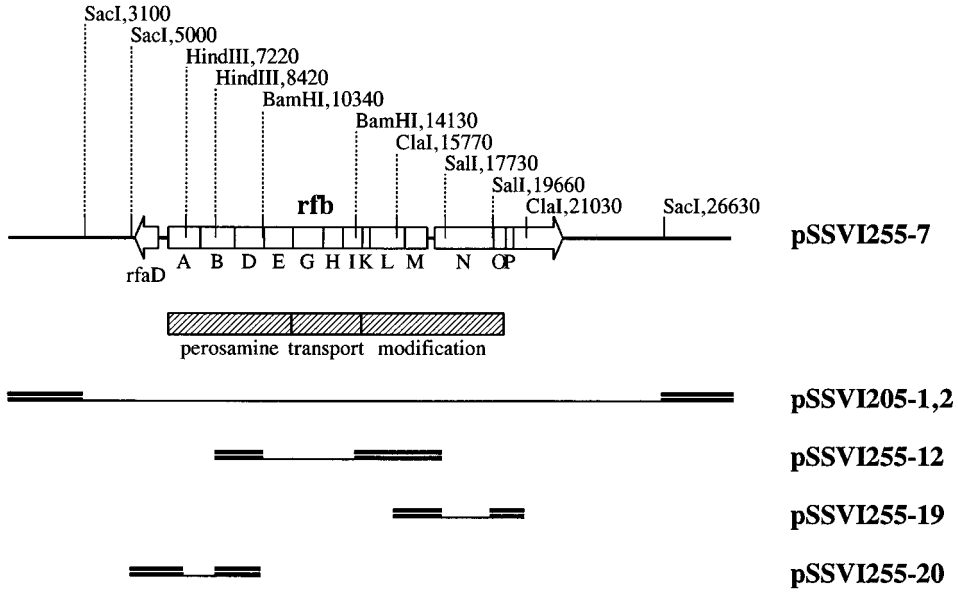

FIG. 2. Deletion plasmids generated from pSSVI255-7. Only restriction sites relevant to the construction of the deletion vectors are shown. The arrows depict the direction of transcription of the *rfaD* gene and rfb_{Inaba} operon, the white boxes delimit the various *rfb* genes, and the striped boxes denote functional regions as inferred from published results (44, 45). The inserts present in the various deletion plasmids are indicated under the corresponding regions of the pSSVI255-7 map. The portions with a thick double line represent homologous regions used for chromosomal integration and excision of vector sequences. The remaining portion (thin line) represents the chromosomal regions deleted from each plasmid.

ing, in both orientations, of the remaining insert into the blunted *Hin*dIII site of pMAK700oriT. Plasmid pSSVI255-12 corresponds to pMAK700oriT bearing the 10.5-kb *Sal*I-*Hin*dIII central fragment of pSSVI255-7, from which the 3.8-kb internal *Bam*HI fragment was deleted. This deletion removes the *rfbEGHI* genes. Gene *rfbE* is the putative perosamine synthetase, whereas *rfbG*, *-H*, and *-I* are presumably involved in transport of the rfb_{Inaba}-encoded components through the outer membrane (44, 45). Plasmids pSSVI255-19 and pSSVI 255-20 were both derived from pSSVI255-7 through subcloning of defined restriction fragments into the high-copy-number vector pMTL22p, deletion of a central region, and further subcloning of the resulting insert into pMAK700oriT. Thus, pSSVI255-19 corresponds to the 5.3-kb *Cla*I fragment (map positions 15770 to 21030 [Fig. 2]) from which the internal 1.9-kb *Sal*I fragment was deleted. The deletion overlaps the *rfbN* gene, which is believed to be involved in the synthesis of the perosamine substituent 3-deoxy-L-glycero-tetronic acid. pSSVI255-20 corresponds to the 5.3-kb *Bam*HI-*Sac*I fragment located at the beginning of the rfb_{Inaba} operon (map positions 5000 to 10340), from which the 1.2-kb *Hin*dIII internal fragment was deleted. This deletion inactivates the *rfbA* and *rfbB* genes, which are directly involved in biosynthesis of the perosamine O-antigen subunit.

These various clones were then transferred into CVD103-HgR and candidate vaccine strain CH3 or CH9 by conjugation. Successful introduction of these deletions was dependent on both the type of deletion and the recipient strain. Indeed, deletion of the entire rfb_{Inaba} operon (as would be obtained from clones pSSVI205-1 and -2) was never successful despite repeated attempts, even when genes for the heterologous O-PS were present. Such extensive deletions are probably lethal to the cell. Other deletion mutants could be obtained for certain strains only (e.g., a 2-kb *Sal*I deletion from pSSVI255-19 was successfully obtained in CVD103-HgR and CH9) but not for others (CH3). Δ*rfbEGHI* mutants arising from the integration of pSSVI255-12 into CH3 and CH9 are referred to as CH13 and CH14, respectively. A corresponding mutant could not be obtained in CVD103-HgR. Δ*rfbN* mutants obtained in CVD103-HgR and CH9 with pSSVI255-19 were designated CH15 and CH17, respectively, whereas Δ*rfbAB* mutants in CVD103-HgR and CH9 constructed with pSSVI255-20 were named CH19 and CH21, respectively (Fig. 3). Since we could not produce a CH3 deletion mutant using pSSVI255-20, a genotypically similar strain, CH22, was constructed by the reverse approach, namely the integration of rfb/rfc_{sonnei} genes carried on plasmid pSSVI201-1 into the

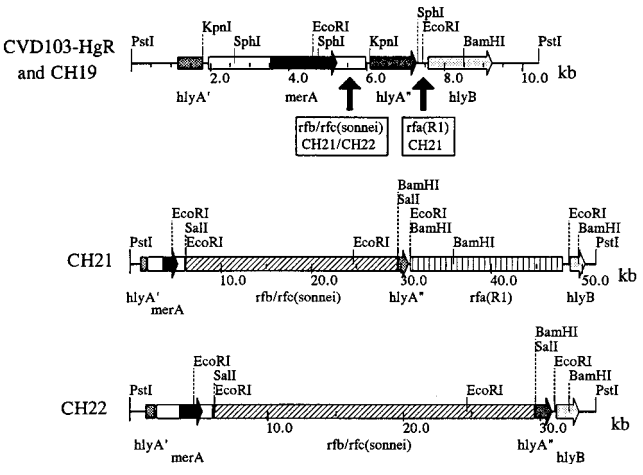

FIG. 3. Genetic structures of CVD103-HgR and CH19, CH21, and CH22 at the *hlyA*::*mer*/*hlyB* loci. The vertical arrows point to the location where the indicated loci were chromosomally inserted. Arrows denote the direction of transcription of the indicated genes. The open boxes represent the entire *mer*-containing fragment inserted into *hlyA*.

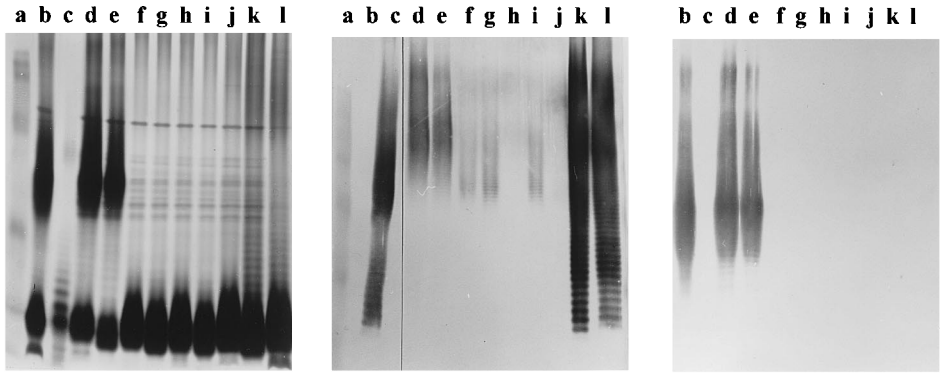

FIG. 4. SDS-PAGE analysis of homologous and/or heterologous O-PS production from recombinant CVD103-HgR derivatives. LPSs were prepared by the miniprep method. (A) Silver stained gel; (B) immunoblot with *S. sonnei*-specific MAb Sh5S; (C) immunoblot with the *V. cholerae* O-PS-specific MAb VCO4. Lanes: a, molecular weight standards; b, CVD103-HgR; c, *S. sonnei* 482-79(pWR105); d, CH3; e, CH9; f, CH13; g, CH14; h, CH15; i, CH17; j, CH19; k, CH21; l, CH22.

chromosome of CH19 (Fig. 3). These deletion strains were genetically characterized by Southern hybridization with probes specific for either the rfb_{Inaba} or rfb/rfc_{sonnei} loci in addition to a probe for the *hlyA* gene, the integration target for the rfb/rfc_{sonnei} locus. The genetic structures of all strains tested conformed to expectations (data not shown).

Expression of homologous and heterologous LPSs in deletion mutants. Figure 4 depicts the expression of *S. sonnei* and Inaba LPSs in the various deletion mutants and their respective parent strains. All tested $\Delta rfb_{\text{Inaba}}$ mutations abolished the expression of Inaba O-PS (Fig. 4A and C, lanes f to l versus lanes b, d, and e). Interestingly, such deletions also affected the expression of *S. sonnei* O-PS to various degrees. As previously reported (65), strains CH3 and CH9 (lanes d and e, respectively) both expressed limited amounts of core-bound *S. sonnei* O-PS (Fig. 4A). When deletions in genes for Inaba O-PS transport/perosamine synthesis or tetronate synthesis were introduced in these strains (CH13/CH14 and CH15, respectively), *S. sonnei* O-PS was expressed in small amounts (lanes f, g, and i of Fig. 4B) of highly polymerized material, which was reminiscent of unbound O-PS similar to that described by Seid et al. (59). In contrast, the deletion specifically affecting perosamine synthesis (strains CH21 and CH22) allowed for the expression of large amounts of core-bound heterologous *S. sonnei* O-PS, which are depicted as typical LPS ladder-like structures in the lower part of the gel (Fig. 4A and B, lanes k and l). Identical patterns were observed for hot phenol-purified LPS preparations of these last strains, demonstrating covalent binding to the LPS core (data not shown).

Physiological properties of deletion mutants. The physiological properties of the genetically defined $\Delta rfb_{\text{Inaba}}$ mutants cultured at 30°C are summarized in Table 2. The phenotypes of the deletion mutants were markedly influenced by growth in various media, whereas that of CVD103-HgR was not. When cultivated at 37°C, all strains, including CVD103-HgR, showed a drastically reduced motility. The inability of CVD103-HgR rough mutants to synthesize the Inaba O-PS following the introduction of the Δ*rfbEGHI* (strain CH15) or Δ*rfbAB* (strain CH19) deletions resulted in similar phenotypes, which were quite different from their isogenic counterparts expressing *S. sonnei* O-PS (CH17, CH21, and CH22). Thus, both CH15 and CH19 were poorly motile, grew mostly as single cells or short filaments, and, most strikingly, spontaneously aggregated in all media tested. Expression of *S. sonnei* O-PS in these strains restored many phenotypic traits inherent to CVD103-HgR, such as motility and growth in nonaggregated, mostly nonfilamentous form. Strain CH13, which contains the Δ*rfbEGHI* deletion in the CH3 background, displayed an intermediate phenotype with limited motility and a modest degree of aggregation. Strain CH22, possessing the Δ*rfbAB* mutation, displayed the phenotype closest to that of CVD103-HgR. Coexpression of the R1 core in CH17 and CH21 resulted in filamentous growth and a diminution of motility.

Further characterization of vaccine candidates CH21 and CH22. After more than 50 generations of growth in LB medium, both strains were found to stably express (>99.9%) *S. sonnei* O-PS. A similar proportion were found to maintain and express the R1 LPS core in CH21. On the other hand, all tested colonies failed to express the *V. cholerae* Inaba O-PS. CH21 and CH22 were indistinguishable from CVD103-HgR with regard to the lack of cholera toxic activity in the Y1-adrenal cell assay, the ability to produce the B subunit of cholera toxin as determined in the GM1 ganglioside-binding assay, and mercury resistance (growth in the presence of 70 to 80 μM $HgCl_2$) (data not shown).

Toxin-coregulated pili, products of the *tcp* regulon, are known to be an important factor for *V. cholerae* adhesion to the intestinal cells. In order to evaluate the expression of *tcpA*, the gene coding for pilin, Western blots of whole-cell extracts of CVD103-HgR, CH21, and CH22 probed with a pilin-specific polyclonal antiserum showed that both CH21 and CH22 produce amounts of pilin similar to those of CVD103-HgR (results not shown).

Sera from mice immunized with killed whole CH22 cells were tested for the presence of *S. sonnei* phase I and CVD103-HgR LPS-specific antibodies. As controls, nonimmune sera or sera from mice immunized with killed whole CVD103-HgR cells were used. As shown in Table 3, immunization with CH22 induced high titers of *S. sonnei* LPS-specific antibodies but no Inaba LPS-specific antibodies. In contrast, sera from mice immunized with CVD103-HgR produced only Inaba LPS-specific antibodies. Sera from control mice did not react with any of the LPS test antigens.

DISCUSSION

The fact that many bacterial enteric pathogens are coendemic in many areas of the world illustrates the need for multivalent vaccines to facilitate immunization programs (68). One approach to the development of such vaccines is to con-

TABLE 2. Phenotypic characterization of CVD103-HgR and Inaba LPS mutants[a]

Strain	Affected function	Medium[b]	Motility[c]	Cellular phenotype		
				Single cells	Filaments	Aggregates[d]
CVD103-HgR	None	CF	+++	+++	−	−
		LB	+++	+++	−	−
		BHI	++	+++	−	−
CH13	O-antigen transport, synthesis	CF	−	++	++	−
		LB	+	+++	+	+
		BHI	−	++	+	+
CH14	O-antigen transport, synthesis	CF	−	++	+++	−
		LB	+	++	+++	+
		BHI	−	+	+++[e]	−
CH15	Perosamine modification	CF	−	++	+++	+++
		LB	+	+	+	+++
		BHI	+	++	+++	++
CH17	Perosamine modification	CF	−	+	+++[e]	−
		LB	++	+	+++	+
		BHI	+	++	+++[e]	−
CH19	Perosamine synthesis	CF	−	+++	+	+++
		LB	+	+++	−	+++
		BHI	+	+++	−	+++
CH21	Perosamine synthesis	CF	−	++	+++[e]	−
		LB	+	++	+++	−
		BHI	−	+	+++[e]	−
CH22	Perosamine synthesis	CF	++	++	++	−
		LB	+++	+++	+	−
		BHI	+	+++	++	−

[a] −, not present; +, present in 1 to 20% of population; ++, present in 20 to 60% of population; +++, present in 60 to 100% of population.
[b] The strains were grown to stationary phase at 30°C in the indicated media. CF, colonization factor broth; BHI, brain heart infusion broth.
[c] Microscopically determined.
[d] Large clusters of adherent cells.
[e] Most filaments consisted of ≥10 cells.

struct live oral attenuated strains capable of expressing protective antigens from multiple etiologic agents. Attempts to construct such strains have relied primarily on the use of attenuated vaccine strains as carriers into which genes coding for purported protective antigens from heterologous strains are introduced. A variety of attenuated carriers have shown promising results in preclinical trials (4, 6). However, human studies with the *S. typhi* Ty21a vaccine strain to present either *V. cholerae* or *S. sonnei* O-PS have conferred only modest protection against experimental challenges (2, 4, 27).

For such bivalent vaccines to be effective, several criteria must be met as follows: (i) the foreign locus and its homologous counterpart in the carrier strain are both expressed in roughly equal amounts; (ii) the expression of the heterologous antigen does not adversely affect critical growth or phenotypic characteristics of the carrier; (iii) coexpression of carrier and heterologous antigens does not result in masking one antigen by the other, thereby down-regulating the immune response; (iv) the ability of the recombinant strain to colonize the intestinal tract is undiminished. We and others have previously demonstrated that the expression of heterologous O-PS on the surface of such carrier strains can adversely affect one or more of these parameters (1, 19, 65). Therefore, it may prove extremely difficult to construct a bivalent vaccine candidate strain capable of expressing and engendering a vigorous immune response against two heterologous O-PS molecules. To optimize the expression of *S. sonnei* O-PS in *V. cholerae*, we previously isolated spontaneous Inaba-deficient mutants of CVD103-HgR derivatives bearing chromosomally integrated *S. sonnei* LPS genetic determinants and showed that the mutants could synthesize large amounts of core-bound *S. sonnei* O-PS on the cell surface (65, 67).

TABLE 3. Antibody response following immunization with *V. cholerae* CH22 or CVD103-HgR

Immunizing strain[a]	Geometric mean antibody titer[b]	
	S. sonnei phase 1 LPS	*V. cholerae* Inaba LPS
None	<10	<10
CH22	3,313 (650–10,200)	<14 (<10–71)
CVD103-HgR	<10	260 (57–730)

[a] Groups of seven mice were immunized intramuscularly at days 0 and 14 with 5×10^7 heat-inactivated cells. A booster dose was given intraperitoneally on day 21. Control mice were not immunized. All mice were sacrificed on day 28.
[b] Sera were tested individually for LPS-specific antibodies with purified *S. sonnei* phase 1 or *V. cholerae* Inaba as coating antigens in an ELISA. Titers are expressed as the geometric means (ranges) of the reciprocals of the highest dilution resulting in an optical density at 405 nm of 0.4.

On the basis of these results, we attempted an alternative approach consisting of the introduction of precise deletions within the rfb_{Inaba} locus to abolish the expression of Inaba O-PS while maintaining that of *S. sonnei* O-PS. This process was complicated by the complexity of this locus, which encodes many proteins involved in O-PS biosynthesis, including transport of precursor O-PS across the cell wall, perosamine synthesis, tetronate synthesis and modification of perosamine, and Inaba/Ogawa serotype interconversion (45).

Attempts to abolish synthesis of Inaba O-PS through deletion of the entire rfb_{Inaba} locus had an apparently lethal effect on the cell, possibly indicating a crucial role for one or more *rfb* gene products. However, at least one gene involved in LPS core biosynthesis (*rfaD*) is apparently located adjacent to the *rfb* locus in *V. cholerae* (45). Therefore, since the deleted fragment (see plasmids pSSVI205-1 and pSSVI205-2 in Fig. 2) may

contain *rfaD* in addition to the complete *rfb* locus, the lethal effect may instead be related to a defect in LPS core biosynthesis. If such is the case, the lethality of this particular deletion in the CH9 background would indicate that the integrated rfa_{R1} locus cannot complement the defect. Deletion of genes involved in O-PS transport and perosamine synthesis (Δ*rfb EGHI*) was also found to be lethal in the CVD103-HgR background, perhaps because of the intracellular accumulation of a toxic intermediate. In contrast, Δ*rfb* deletions in the same background affecting biosynthesis of either perosamine (Δ*rfb AB*) or its tetronate substituent (Δ*rfbN*, which is thought to be involved in the synthesis of 3-deoxy-L-glycero-tetronic acid) were nonlethal, although they altered cellular morphology and motility. The cellular aggregation displayed by these deletion mutants, which was also observed for spontaneous Inaba LPS mutants of CVD103-HgR (65), can most likely be attributed to the increased hydrophobicity of their cell surfaces, as previously shown for rough *V. cholerae* strains (30). A similar effect was noted by Manning et al. (45), who reported that strains of *V. cholerae* deficient in perosamine biosynthesis were poorly motile because of abnormal flagella. Such alterations are not unexpected given the facts that (i) LPS is known to be present on the surface of the polar flagellum of *V. cholerae* (21) and (ii) the expression of complete LPS is known to be essential for maintenance of structural and functional outer membrane integrity (20, 38, 48, 54, 57).

A finding with important potential implications was that Δ*rfbEGHI* mutants were viable when the rfb/rfc_{sonnei} locus, alone or together with the rfa_{R1} locus, was expressed. This would imply some degree of complementation by the corresponding *S. sonnei* genes. However, in such strains (CH13 and CH14), the modest amount of *S. sonnei* O-PS present on the cell surface, possibly in a form unbound to the LPS core, demonstrates only partial complementation of the transport and linkage defects. It is possible that *V. cholerae* and *S. sonnei* utilize somewhat different mechanisms by which O-PS is translocated to the cell surface or that the corresponding rfb/rfc_{sonnei} genes are inefficiently expressed. A further possibility is that the Δ*rfbEGHI* deletion interferes with the expression of downstream gene(s). In favor of the latter possibility is the observation that the Δ*rfbN* mutant CH17 affected in tetronate biosynthesis displays a phenotype very similar to those of the Δ*rfbEGHI* mutants. The importance of preserving these functions is illustrated by the fact that expression of core-bound *S. sonnei* O-PS and maintenance of normal cellular morphology and motility occurred only when genes involved in perosamine synthesis were specifically affected. Most importantly, disruption of the *rfbAB* genes does not interfere with the transport and binding of the heterologous O-PS to the host strain LPS core, thus allowing for full-length hybrid LPS to be synthesized. These observations imply that monovalent vaccines based on the expression of a heterologous O-PS in a vaccine carrier can be achieved only through careful choice of the mutation-disabling synthesis of homologous O-PS. Thus, *V. cholerae* Δ*rfbAB* deletion mutants appear to be the best-suited candidate carriers for the expression of heterologous O-PS antigens.

The expression of the R1 core is not necessary for obtaining full-length LPS composed of *S. sonnei* O-PS in *V. cholerae*, as was initially expected (66). The simultaneous expression of the R1 and *V. cholerae* LPS cores, as, for example, in strains CH5 (65), CH9, CH17, and CH21, results in loss of motility and atypical filamentous growth. Thus, the insertion of the heterologous core molecules into the outer membrane can adversely affect its function and integrity. However, these features may under certain circumstances be an advantage, since motility is believed to play some role in the pathogenesis of *V. cholerae* infections, for nonmotile mutants were found to be less pathogenic (31). Moreover, recently described spontaneous nonmotile mutants of *V. cholerae* O139 and El Tor Inaba vaccine candidates were found to colonize the gut as efficiently as wild-type strains and to engender a comparable immune response (8, 62). In addition, individual M cells of Peyer's patches are known to be able to bind multiple bacteria (23). Therefore, providing that filamentous growth of, e.g., CH21 still occurs in the human gut, the immune response could conceivably be potentiated by M cell uptake of entire bacterial filaments instead of just individual cells, thereby increasing the amount of sampled antigen.

Cultivation conditions were also found to play a pronounced role in expression of the phenotypes of CVD103-HgR and recombinant strains, further demonstrating the complexity of strategies aiming at optimizing the proper expression of foreign surface antigens in carrier strains.

As for the CVD103-HgR parent, extensive characterization of strains CH21 and CH22 showed lack of cholera toxin activity, production of the nontoxic B subunit of the cholera toxin and of TcpA pilin, and a mercury-resistant phenotype. The phenotypes expressed by these strains were extremely stable, which we attribute to the fact that the foreign genes are integrated into the chromosome. Therefore, both CH21 and CH22 fulfill general requirements for production scale-up. Preliminary studies with humans to evaluate the safety and immunogenicity of these strains are planned for the near future.

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