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Heterologous Antigen Expression in *Vibrio cholerae* Vector Strains

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Live attenuated vector strains of *Vibrio cholerae* were derived from Peru-2, a Peruvian El Tor Inaba strain deleted for the cholera toxin genetic element and *attRS1* sequences, which was developed as a live, oral vaccine strain. A promoterless gene encoding the Shiga-like toxin I B subunit (*slt*-IB) was inserted in the *V. cholerae* virulence gene *irgA* by in vivo marker exchange, such that *slt*-IB was under transcriptional control of the iron-regulated *irgA* promoter. *slt*-IB was also placed under transcriptional control of the *V. cholerae* heat shock promoter, *htpGp*, and introduced into either the *irgA* or *lacZ* locus, or both loci, on the chromosome of Peru-2, generating JRB10, JRB11, or JRB12, respectively. A new technique was used to perform allelic exchange with *lacZ*. This method uses plasmid p6891MCS, a pBR327 derivative containing cloned *V. cholerae lacZ*, to insert markers of interest into the *V. cholerae* chromosome. Recombinants can be detected by simple color screening and antibiotic selection. In vitro measurements of Slt-IB produced by the vector strains suggested that expression of Slt-IB from the *irgA* and *htpG* promoters was synergistic and that two copies of the gene for Slt-IB increased expression over a single copy. The *V. cholerae* vectors colonized the gastrointestinal mucosa of rabbits after oral immunization, as demonstrated by very high serum antibody responses to *V. cholerae* antigens. Comparison of the serologic responses to the B subunit of cholera toxin (CtxB) following orogastric inoculation either with the wild-type C6709 or with Peru-10, a strain containing *ctxB* regulated by *htpGp*, suggested that both the cholera toxin and heat shock promoters were active in vivo, provoking comparable immunologic responses. Orogastric inoculation of rabbits with vector strains evoked serum immunoglobulin G (IgG) responses to Slt-IB in two of the four strains tested; all four strains produced biliary IgA responses. No correlation was observed between the type of promoter expressing *slt*-IB and the level of serum IgG or biliary IgA response, but the vector strain containing two copies of the gene for *slt*-IB evoked greater serum IgG responses than strains containing a single copy, consistent with the increased expression of Slt-IB from this strain observed in vitro. A comparison of the serum and biliary antibody responses to Slt-IB expressed from *htpGp* versus CtxB expressed from the same promoter suggested that CtxB is a more effective orally delivered immunogen.

Vibrio cholerae infection remains a major cause of morbidity and mortality from diarrheal disease in many developing countries, reinforcing the need for effective vaccines to prevent cholera. Naturally acquired infection with *V. cholerae*, or oral inoculation of human volunteers, leads to long-term protection against subsequent infection (23), suggesting that it should be feasible to develop an effective oral vaccine against this pathogen. Several live, oral attenuated vaccine strains of *V. cholerae* have been shown to confer relatively high levels of protection after a single dose, but they vary in efficacy and immunogenicity and may produce some residual symptoms in vaccinees (20, 24, 27, 41).

All recently developed live oral cholera vaccine strains have deletions in the genes encoding the subunits of cholera toxin, the most important virulence factor for *V. cholerae* in producing diarrhea. The cholera toxin (*ctx*) genes are arranged in an operon within a larger cholera toxin (CTX) genetic element, which is composed of a core (containing *ctxAB* [14], the toxin-encoding genes *zot* [2] and *ace* [42], and a colonization factor gene, *cep* [32]) as well as two or more flanking copies of a directly repeated sequence, RS1, which contain at their termini a 17-bp end-repeat sequence. The end-repeat element shares 17 of 18 bp with a sequence on the *V. cholerae* chromosome called *attRS1*, which represents the chromosomal insertion site for the CTX genetic element (32). Strains deleted for *attRS1* are unable to reacquire the CTX genetic element by site-specific recombination (32). Recently, several different El Tor O1 strains carrying *attRS1* deletions have been constructed and evaluated as attenuated, live oral cholera vaccines (41).

An additional virulence factor for *V. cholerae* is IrgA, the major iron-regulated outer membrane protein of this organism (16). Iron is an important in vivo signal controlling expression of many bacterial virulence factors; in vivo, *V. cholerae* expresses proteins that may correspond to proteins shown to be iron regulated in vitro (36). A mutation in *irgA* causes a 100-fold defect in virulence of classical strains of *V. cholerae* and a 10-fold decrease in colonization in an infant mouse model (16).

Mucosal pathogens continue to account for a disproportionate share of morbidity and mortality in the developing world due to infections of the gastrointestinal, respiratory, and genitourinary systems. Many of these diseases are those for which current vaccines are ineffective. A common mucosal immune system responds to binding of antigens to the gastrointestinal surface of M cells by translocation of antigens across the epithelium to stimulate lymphocytes that circulate and populate the mucosal tissues of the body as immunoglobulin A (IgA)-secreting plasma cells (44). The selective adherence of *V. cholerae* to the M cells of the gastrointestinal tract makes it a

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype[a]	Reference or source
V. cholerae O1 strains		
C6709	El Tor, Inaba, wild type, Sm^r	34
Peru-2	C6709 Δ*attRS1* Sm^r	34
Peru-8	C6709 Δ*attRS1* Δ*irgA irgA*::*irgP*→*slt*-IB Sm^r	This study
Peru-10	C6709 Δ*attRS1* Δ*irgA* Δ*recA irgA*::*irgP*→*slt*-IB *recA*::*htpGp*→*ctxB* Sm^r	This study
JRB10	C6709 Δ*attRS1* Δ*irgA irgA*::*irgP, htpGp*→*slt*-IB Sm^r	This study
JRB11	C6709 Δ*attRS1 lacZ*::*htpGp*→*slt*-IB Sm^r	This study
JRB12	C6709 Δ*attRS1* Δ*irgA irgA*::*irgP, htpGp*→*slt*-IB *lacZ*::*htpGp*→*slt*-IB Sm^r	This study
N16961	El Tor, Inaba, wild type	Laboratory collection
E. coli strains		
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*R6K Km^r	28
SY327	F^- Δ(*lac-pro*) *nalA recA56 araD argE*(Am) *rif*	13
SY327λ*pir*	λ*pir*R6K derivative of SY327	28
Plasmids		
pSBC53	pKK233-2 (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) with a promoterless gene for the B subunit of Slt-I cloned between the *Eco*RI and *Hin*dIII sites, Ap^r	1
pSBC62	pSBC53 with *Eco*RI site eliminated, Ap^r	This study
pSAB18	pUC18 with a 533-bp fragment of upstream *V. cholerae irgA* cloned within the *Hin*dIII and *Sph*I sites and a 1.5-kbp fragment of downstream *irgA* cloned within the *Sac*I site, Ap^r	5
pSAB3	*Sal*I-*Sma*I fragment from pSBC62, encoding *slt*-IB, into *Sal*I and *Sma*I sites of pSAB18, Ap^r	This study
pJRB28	pSAB3 with deletion of internal *Sal*I site, Ap^r	This study
pVC100	pUC19 with an *Eco*RI-*Dra*I fragment containing the *V. cholerae htpG-toxR* intergenic region and a *toxR*::*lacZ* transcriptional fusion, cloned into the pUC19 *Hin*cII site	31
pPAC4	592-bp *Bam*HI-*Xba*I fragment of pVC100, containing *htpGp*, inserted upstream of *slt*-IB in pJRB28, Ap^r	This study
pJRB29	3.4-kbp *Hin*dIII-*Eco*RI fragment of pPAC4 containing the *irgA* deletion–*irgP, htpGp*→*slt*-IB substitution ligated with *Sal*I linkers into the *Sal*I site of pUC18, Ap^r	This study
pCVD442	Suicide vector composed of the *mob*, *ori*, and *bla* regions from pGP704 and the *sacB* gene of *B. subtilis*, Ap^r	10
pJRB31	3.4-kbp *Sal*I fragment of pJRB29 ligated into the *Sal*I site of pCVD442, Ap^r	This study
pCG698	8-kbp *Sau*3A fragment of *V. cholerae*, encoding *lacZ*, cloned into the *Bam*HI site of pBR327, Ap^r	This study
p6891MCS	pCG698 with multiple cloning site inserted in the *Kpn*I site within *lacZ*, Ap^r	This study
pJRB32	1,066-bp *Bam*HI-*Sma*I fragment of pJRB29, containing *htpGp*→*slt*-IB, within *Bgl*II and *Stu*I sites in p6891MCS, Ap^r	This study
pAR62	*V. cholerae* chromosomal DNA, flanking a defined deletion of the entire CTX element, cloned in pLAFR2, Tc^r	32
pSAB24	pCVD442 containing a deletion-substitution of *irgA*::*irgP*→*slt*-IB, Ap^r	5
pJM84.1	Contains *ctxB* under control of *htpGp* cloned into *Xba*I deletion in *V. cholerae recA* locus	43

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance, Tc^r, tetracycline resistance.

potent stimulus to the common mucosal immune system (9, 29, 37). We hypothesize, therefore, that *V. cholerae* may be a particularly effective organism to use as a live, oral attenuated vaccine vector that can deliver heterologous antigens stimulating a common mucosal immune response.

We have previously described the introduction of an *irgA* mutation into classical *V. cholerae* O395 which was deleted for *ctxA*, and the insertion of a promoterless gene for the Shiga-like toxin I B subunit (*slt*-IB) into the *irgA* deletion, thus producing a live attenuated vector strain of *V. cholerae* that contains *slt*-IB under the transcriptional control of the iron-regulated *irgA* promoter (5). Because the seventh-pandemic strain is of the El Tor biotype (38, 40), and since the tandem configuration of the cholera toxin genetic element in the El Tor biotype allows for *attRS1* deletions (26, 32), we now describe the development of oral, live attenuated vector strains of the El Tor biotype. We also demonstrate a new technique for rapid in vivo marker exchange, using a system for inserting markers of interest into the *V. cholerae lacZ* gene. Finally, we compare different promoters for in vivo expression of Slt-IB and examine systemic and mucosal immune responses to the antigens in different vaccine strains, using an animal model.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1; constructions of strains and plasmids are schematically depicted in Fig. 1 to 3. Standard plasmid cloning vectors pUC18 and pBR327 were from laboratory stocks.

Media. All strains were maintained at −70°C in Luria broth (LB) medium (35) containing 15% glycerol. LB medium or AKI medium (22) with or without the

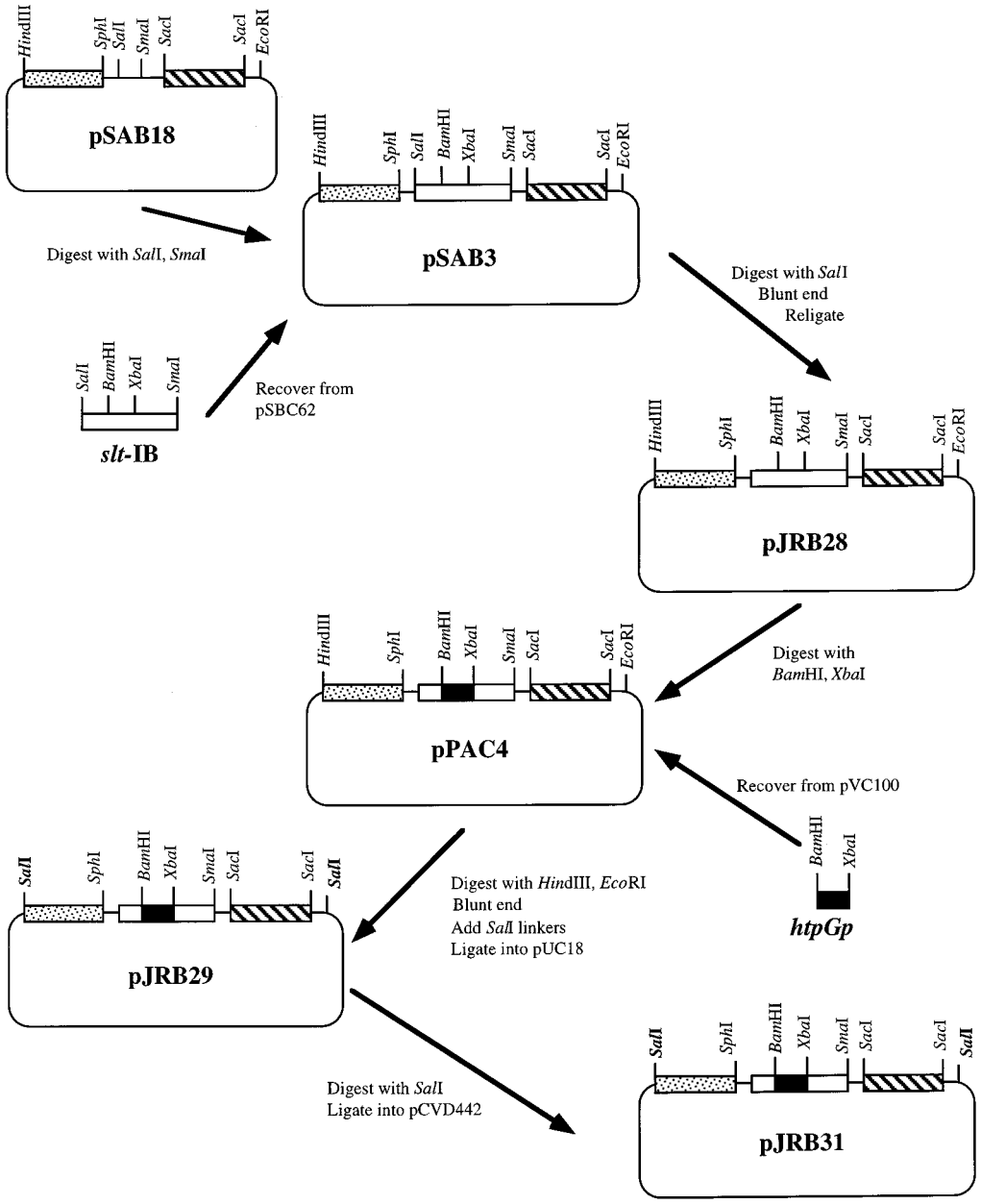

FIG. 1. Construction of *irgA* deletion derivatives used in this study. The upstream *irgA* fragment is indicated by a stippled bar, the downstream *irgA* fragment is indicated by a hatched bar, the *slt*-IB subunit is indicated by an open bar, and the *htpGp* fragment is indicated by a solid bar. Plasmids and chromosomal fragments are not drawn to scale.

addition of the iron chelator 2,2-dipyridyl (final concentration, 0.2 mM) was used for growth in low- or high-iron conditions, respectively. Ampicillin (100 μg/ml), streptomycin (100 μg/ml), or X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 80 μg/ml; International Biotechnologies, Inc., New Haven, Conn.) was added as appropriate.

Genetic methods. Isolation of plasmid and bacterial chromosomal DNA, restriction enzyme digestions, agarose gel electrophoresis, and Southern hybridization of DNA separated by electrophoresis were performed according to standard molecular biologic techniques (35). GeneScreen Plus hybridization transfer membranes (DuPont Biotechnology Systems, NEN Research Products, Boston, Mass.) were used according to the manufacturer's protocols for Southern hybridization. DNA sequencing was performed by using a Sequenase DNA sequencing kit (United States Biochemical Corporation, Cleveland, Ohio).

Plasmids were transformed into *Escherichia coli* strains by standard techniques or were electroporated into *V. cholerae*, using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) as instructed by the manufacturer, and modified for electroporation into *V. cholerae* as previously described (15). Electroporation conditions were 2,500 V at 25-μF capacitance, producing time constants of 4.7 to 4.9 ms.

DNA restriction endonucleases, T4 DNA ligase, calf intestinal alkaline phosphatase, and the Klenow fragment of DNA polymerase I were used according to the manufacturers' specifications. Restriction enzyme-digested chromosomal and plasmid DNA fragments were separated on 1% agarose gels; required fragments were cut from the gel under UV illumination and purified by the freeze-squeeze technique (39). DNA fragments used as probes were radiolabeled with [α-^{32}P]dCTP, using a random priming labeling kit (Prime Time "C" Oligonucleotide Labelling Biosystem; International Biotechnologies, Inc.).

Construction of plasmids. A DNA segment encoding the promoterless B subunit of Shiga-like toxin I (*slt*-IB) was recovered as a *Sal*I-*Sma*I fragment from plasmid pSBC62. This fragment was introduced into the unique *Sal*I and *Sma*I sites of pSAB18 (5) such that *slt*-IB was under the transcriptional control of the *irgA* promoter (*irgP*) on the upstream *irgA* fragment, yielding plasmid pSAB3 (Fig. 1). The *Sal*I site of pSAB3 was deleted by digestion with *Sal*I, treatment with the Klenow fragment of DNA polymerase I, and religation, to create

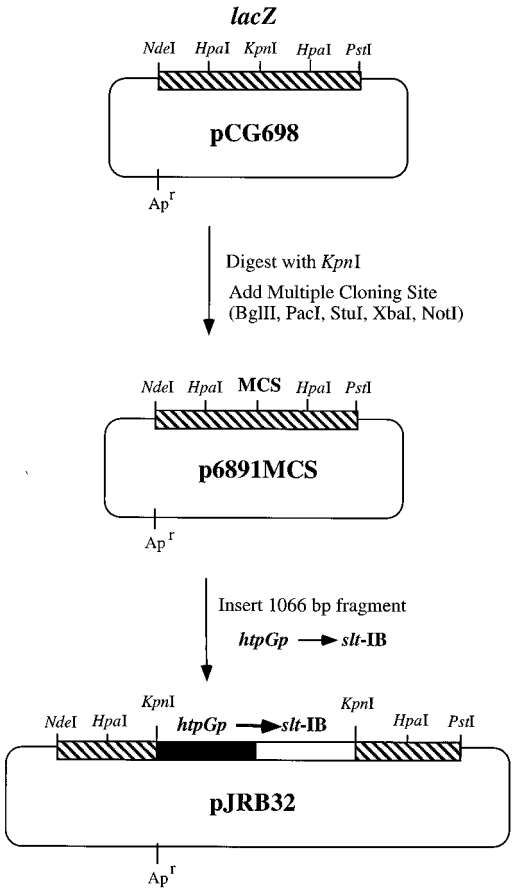

FIG. 2. Construction of pJRB32. *V. cholerae lacZ* is indicated by a hatched bar, the *slt*-IB subunit is indicated by an open bar, and the *htpGp* fragment is indicated by a solid bar. Plasmids and chromosomal fragments are not drawn to scale. MCS, multiple cloning site.

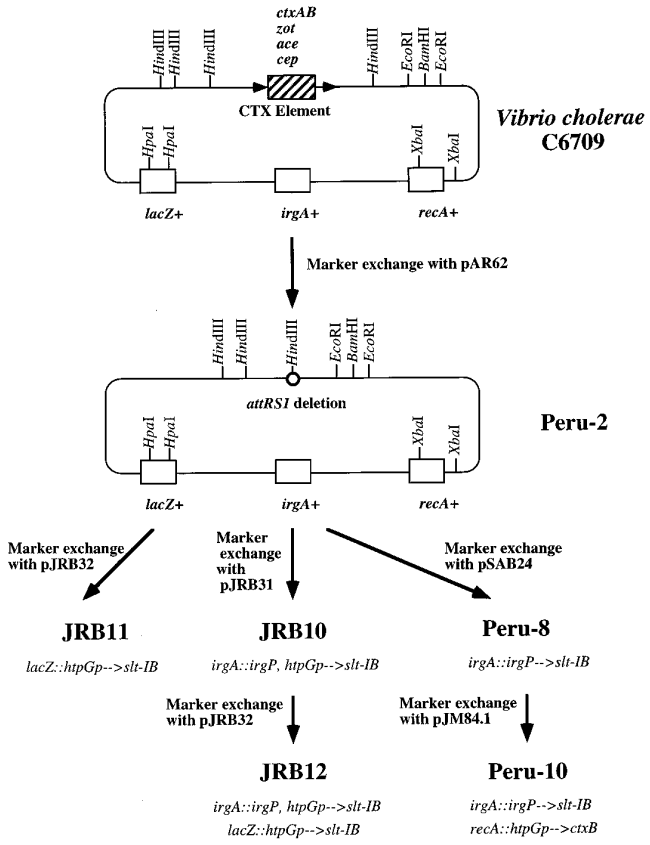

FIG. 3. Construction of *V. cholerae* C6709 *attRS1* deletion strains and *slt*-IB derivatives. See text for details.

plasmid pJRB28. The 592-bp *Bam*HI-*Xba*I fragment of plasmid pVC100 (31), containing the promoter of *V. cholerae htpG* (*htpGp*), was ligated into the *Bam*HI and *Xba*I sites of pJRB28, so that *slt*-IB is under the control of both *irgP* and *htpGp*, to produce plasmid pPAC4.

pPAC4 was digested with *Hin*dIII and *Eco*RI, and the 3.4-kb DNA fragment containing the *irgA* deletion–*htpGp*→*slt*-IB substitution was blunt ended by the Klenow fragment of DNA polymerase I. Following ligation to *Sal*I linkers and digestion with *Sal*I, the fragment was ligated into the unique *Sal*I site of pUC18, yielding plasmid pJRB29. The construction of plasmid pJRB29 was verified by restriction enzyme digestion and double-stranded DNA sequencing. pJRB29 was digested with *Sal*I, and the 3.4-kb DNA fragment was ligated into the unique *Sal*I site of pCVD442, to give plasmid pJRB31, and propagated in the permissive strain SM10λ*pir*. Plasmid pCVD442 is a suicide vector containing the *pir*-dependent R6K replicon, ampicillin resistance, and the *sacB* gene from *Bacillus subtilis* (10).

Plasmid pCG698 contains an 8-kb *Sau*3A fragment of *V. cholerae* chromosomal DNA, encoding *lacZ*, cloned into the *Bam*HI site of pBR327 (Fig. 2). A plasmid DNA library from the classical *V. cholerae* strain 569B (26) was transferred to the Lac$^-$ *E. coli* strain SY327. Transformants were plated onto LB agar with 50 μg of X-Gal, a chromogenic substrate for β-galactosidase, per ml, allowing visualization of enzyme activity. Four blue Lac$^+$ colonies were picked, and plasmids were isolated and characterized by restriction analysis and retransformation into SY327. All four plasmids conferred a Lac$^+$ phenotype and had identical restriction patterns, suggesting a single clone that was named pCG698. Using the *V. cholerae lacZ* sequence (30), a multiple cloning site was inserted within the unique *Kpn*I site in *lacZ* as follows. The multiple cloning site was created by using two oligonucleotides (5′-CAGATCTTAATTAAGGCCTCTAGAGCGGCCGCGGTAC-3′ and 5′-CGCCGGCCGCTCTAGAGGCCTTAATTAAGATCTGGTAC-3′) that, when annealed, contained the sites for *Bgl*II, *Pac*I, *Stu*I, *Xba*I, and *Not*I and produced *Kpn*I-compatible terminal overhangs. The oligonucleotides were annealed by heating to 65°C and slowly cooling to 25°C and were ligated to *Kpn*I-digested plasmid pCG698. The ligation reaction was transformed into competent *E. coli* SY327λ*pir* and plated on LB medium containing 50 μg each of ampicillin and X-Gal per ml. A white colony was picked, and the introduction of the multiple cloning site was confirmed by restriction digestions and sequencing. The resulting plasmid was named p6891MCS. The 1,066-bp *Bam*HI-*Sma*I fragment of pJRB29, containing *htpGp*→*slt*-IB, was inserted into the unique *Bgl*II and *Stu*I sites within the multiple cloning site in p6891MCS to create pJRB32.

Construction of Peru-2, Peru-8, and Peru-10. The starting strain for vaccine constructions was Peru-2, a *V. cholerae* El Tor Inaba strain deleted for *attRS1* sequences and the entire CTX genetic element (34, 41). The construction of *attRS1* deletion strains has been described in detail for the development of live, attenuated O139 strains (43) and is diagrammed in Fig. 3. A method of in vivo marker exchange was used to insert a deletion-substitution of *irgA* containing *slt*-IB into the chromosome of Peru-2 such that *slt*-IB is under the control of the *irgA* promoter, as previously described for the classical *V. cholerae* strain O395 (5), yielding Peru-8. To prevent reacquisition of the CTX genetic element from toxigenic strains in nature by homologous recombination, *ctxB* under the control of *htpGp* was inserted into a deletion of the *recA* gene, as previously described for O139 strains (43), generating Peru-10.

Construction of JRB10. The *irgA* allele of Peru-2 was replaced with the *irgA* deletion containing *htpGp*→*slt*-IB as follows. Plasmid pJRB31 was crossed into Peru-2, with selection for ampicillin and streptomycin. Doubly resistant colonies contained pJRB31 integrated into the chromosome by homologous recombination involving either the upstream or downstream fragment of *irgA* on pJRB31, with creation of a merodiploid state. One such colony was grown overnight in LB medium without ampicillin selection, then plated on LB medium with 10% sucrose but without NaCl, and grown at 30°C for 30 h, thereby selecting for clones that had deleted the integrated *sacB* gene (3). Sucrose-resistant colonies that are ampicillin susceptible have either reexcised the plasmid to yield the parent Peru-2 or resolved the merodiploid state to replace the *irgA* locus in Peru-2 with the *irgA* deletion–*htpGp*→*slt*-IB fragment from pJRB31. Approximately 20% of sucrose-resistant colonies that were ampicillin susceptible had undergone marker exchange. One of these colonies was further purified and named JRB10 (Fig. 3). Confirmation of the proper constructions in JRB10 was obtained by Southern hybridization of restriction enzyme-digested chromosomal DNA that was probed with several different DNA fragments to verify the expected deletion in *irgA*, as well as the introduction of *htpGp*→*slt*-IB within the deleted *irgA* segment (data not shown).

Construction of JRB11 and JRB12. *htpGp→slt*-IB was cloned within the *V. cholerae lacZ* gene to produce pJRB32, as shown in Fig. 2. *V. cholerae* strains were transformed with this plasmid and grown overnight in the absence of ampicillin selection and then plated on medium containing X-Gal. Strains undergoing marker exchange between pJRB32 and the chromosomal copy of *lacZ* were white on X-Gal medium and ampicillin susceptible. Using this procedure, *htpGp→slt*-IB was inserted into *lacZ* on the chromosomes of Peru-2 and JRB10 to create JRB11 and JRB12, respectively (Fig. 3). The expected insertions in *lacZ* within JRB11 and JRB12 were confirmed by Southern hybridization (data not shown).

Preparation of periplasmic extracts. Periplasmic extracts were prepared from exponentially growing cells as previously described (21). *V. cholerae* strains were grown in AKI medium, and *Shigella dysenteriae* 60R was grown in LB medium, with or without dipyridyl to create low- or high-iron conditions, respectively.

Immunodetection of StxB production. The amount of StxB present in periplasmic extracts was quantitated with an enzyme-linked immunosorbent assay (ELISA) developed for the detection of Shiga toxin and modified for detection of purified StxB (6, 11).

Intestinal colonization of suckling mice. Three- to four-day-old suckling CD1 mice were orally inoculated with approximately 10^6 organisms of each vaccine strain to be tested. Animals were sacrificed 20 h after inoculation. The entire intestine was homogenized and plated on selective medium, and CFU were counted.

Orogastric colonization of rabbits. Orogastric colonization of rabbits was performed as previously described (8). *V. cholerae* strains were grown overnight in LB medium, pelleted, washed in phosphate-buffered saline (PBS), and resuspended in fresh LB medium to a concentration of 10^9 organisms per ml. Male New Zealand White rabbits (approximately 2.5 kg in weight) were fasted overnight and sedated. Gastric acid was neutralized with 50 mg of cimetidine per kg intravenously at time zero. Fifteen milliliters of 5.0% $NaHCO_3$ was given by gastric tube at 15 and 30 min; the second dose was followed by the *V. cholerae* inoculum in 15 ml of LB medium. At 60 min, 2 ml of tincture of opium was given intraperitoneally. Serum was drawn on days 0, 14, and 21; bile was obtained on day 21 to estimate mucosal secretory IgA.

Rabbit serum and mucosal antibodies to *V. cholerae* antigens. Serum vibriocidal antibody titers were measured in a microassay as follows. Fifty-microliter aliquots of serial twofold dilutions of the test sera in 0.15 M saline (1:25 to 1:25,600) were placed in the wells of sterile 96-well tissue culture plates; 50 μl of a 10^8-CFU/ml culture of *V. cholerae* N16961 in 0.15 M saline with 22% guinea pig complement (Gibco BRL Life Technologies, Gaithersburg, Md.) was added to the serum dilutions, and the mixtures were incubated for 1 h at 37°C. One hundred and fifty microliters of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) was added to each well, and the wells were incubated for approximately 2.75 h at 37°C. The optical density at 600 nm was then measured; the titer is calculated as the dilution of serum causing 50% reduction in optical density compared with wells containing preimmune serum (day 0).

Rabbit anti-cholera toxin B subunit IgG and IgA were measured as follows. Microtiter plates were coated with 10 μg of type III gangliosides (Sigma Chemical Co., St. Louis, Mo.) per ml in 60 mM sodium carbonate buffer (pH 10) and then blocked with postcoating buffer (Boehringer Mannheim Corporation, Indianapolis, Ind.). Wells were incubated with 0.5 μg of purified CtxB per ml in postcoating buffer. Serial twofold dilutions of serum and bile samples in postcoating buffer were added to the microtiter wells. Serum anti-CtxB IgG was assayed by using goat anti-rabbit IgG conjugated to alkaline phosphatase (ICN Biochemicals, Inc., Costa Mesa, Calif.). Bile anti-CtxB IgA was determined by using goat antiserum against rabbit IgA (alpha chain specific) (Sigma) and then rabbit anti-goat IgG conjugated to alkaline phosphatase (Sigma). Reactions were developed with 2 mg of *p*-nitrophenyl phosphate (Boehringer Mannheim) per ml, diluted in 1 M Tris buffer (pH 8.0), and the optical density at 405 nm was measured. The titer is calculated as the highest dilution of test serum producing an optical density greater than that of the preimmune serum (day 0).

Serum antilipopolysaccharide (anti-LPS) IgG was determined as follows. Microtiter plates were coated with 5 μg of *V. cholerae* Inaba 569B LPS (List Biological Laboratories, Inc., Campbell, Calif.) per ml in 60 mM sodium carbonate buffer (pH 9.8) and then blocked with postcoating buffer. Serial twofold dilutions of serum samples in postcoating buffer were added to the microtiter wells. Serum anti-LPS IgG was assayed by using goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma). Reactions were developed with 2 mg of *p*-nitrophenyl phosphate per ml diluted in 1 M Tris buffer (pH 8.0), and the optical density at 405 nm was measured. The titer is calculated as the highest dilution of test serum producing an optical density greater than that of the preimmune serum (day 0).

Rabbit serum and mucosal antibodies to Slt-IB. Microtiter plates were coated with mouse monoclonal antibodies to Slt-IB and then blocked with PBS–5% nonfat dry milk. Each well was incubated with 0.1 μg of purified Slt-IB. Serial twofold dilutions of serum and bile samples in PBS–Tween–5% milk were added to the microtiter wells. Serum anti-Slt-IB IgG was assayed by using goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma). Bile anti-Slt-IB IgA was determined by using goat antiserum against rabbit IgA (alpha chain specific) and then rabbit anti-goat IgG conjugated to alkaline phosphatase. Reactions were developed with 2 mg of *p*-nitrophenyl phosphate per ml diluted in 1 M Tris buffer (pH 8.0), and the optical density at 405 nm was measured. The titer is calculated as the highest dilution of test serum producing an optical density greater than that of both the preimmune serum from the test animal (day 0) and the serum from the rabbit immunized with the control *V. cholerae* strain, C6709, drawn on the same day (day 14 or 21).

TABLE 2. Production of Slt-IB by ELISA in periplasmic extracts from various strains in high- and low-iron media

Strain	Amt (ng/ml/OD_{600})[a]	
	High-iron medium	Low-iron medium
S. dysenteriae 60R	36 (18–70)	125 (45–147)
V. cholerae		
C6709	0	0
Peru-10	0	11 (5–16)
JRB10	27 (15–51)	37 (35–42)
JRB11	6 (3–11)	8 (4–17)
JRB12	52 (44–64)	37 (31–47)

[a] Mean (range) of three independent measurements. OD_{600}, optical density at 600 nm.

RESULTS

Construction of vaccine strains. Oral, live attenuated vaccine strains of *V. cholerae* were derived from Peru-2, a Peruvian El Tor strain deleted for *attRS1* and the entire CTX genetic element (34, 41) (Fig. 1 to 3). A promoterless gene for the Shiga-like toxin I B subunit (*slt*-IB) was placed under transcriptional control of the iron-regulated *irgA* promoter, and this construct was introduced into *irgA* on the chromosome of Peru-2, to produce strain Peru-8. Allelic exchange was performed with derivatives of the suicide vector plasmid pCVD442, which contains the *sacB* gene from *B. subtilis* and allows positive selection for loss of plasmid sequences upon exposure to sucrose. To prevent reacquisition of the CTX genetic element from toxigenic strains in nature by homologous recombination, *ctxB* under the control of the *V. cholerae* heat shock promoter, *htpGp*, was inserted into a deletion of the *recA* gene, to create Peru-10.

slt-IB was also placed under transcriptional control of *htpGp* and introduced into either the *irgA* or *lacZ* locus, or both loci, on the chromosome of Peru-2, generating JRB10, JRB11, or JRB12, respectively. A new technique was used to perform allelic exchange with *lacZ*. This method uses plasmid p6891MCS, a pBR327 derivative containing cloned *V. cholerae lacZ*, to insert markers of interest into the *V. cholerae* chromosome. Recombinants can be detected by simple color screening on X-Gal agar and antibiotic selection.

These oral vectors allowed a comparison of the expression and immunologic response to Slt-IB under control of either the *irgA* promoter or the heat shock promoter, *htpGp*, as well as determining the immunological effect of the presence of one versus two chromosomal copies of the gene encoding Slt-IB.

Expression of Slt-IB by *V. cholerae* vector strains in vitro. The expression of Slt-IB in vector strains was quantified using a sandwich ELISA. As shown in Table 2, in vitro expression of Slt-IB in periplasmic extracts from Peru-10 was negatively iron regulated, directed by the iron-regulated *irgA* promoter. The expression of Slt-IB from *htpGp* in both low- and high-iron media (JRB11) was comparable to that from the *irgA* promoter (Peru-10) in low-iron medium. The production of Slt-IB in periplasmic extracts from JRB10, containing *irgP, htpGp→slt*-IB, in both low- and high-iron media was approximately threefold greater than that maximally expressed from *irgP* in Peru-10 or *htpGp* in JRB11, suggesting that expression of Slt-IB from the tandem *irgA* and *htpG* promoters in JRB10 was syn-

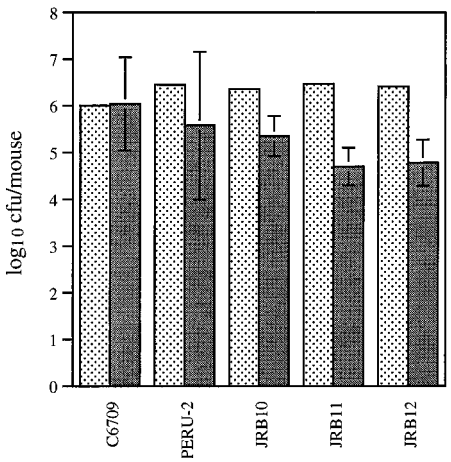

FIG. 4. Intestinal colonization of suckling mice with various *V. cholerae* strains. ▨, organisms administered; ▩, organisms recovered.

ergistic. A comparison of JRB10 and JRB11 with JRB12 suggested that two copies of the gene encoding Slt-IB increased expression over a single copy, at least under high-iron conditions.

Intestinal colonization of suckling mice with *V. cholerae* vector strains. Intestinal colonization of infant mice correlates with the colonization and immunogenicity of live cholera vaccines in humans (41). Vector strains JRB10, JRB11, and JRB12 were only slightly reduced in colonization of the small intestines of suckling mice relative to the parental strains C6709 and Peru-2 (Fig. 4), suggesting that the strains would be immunogenic in humans if used as live vaccine vectors, as previously shown (41).

Serum IgG and bile IgA antibody responses to *V. cholerae* antigens. The immunogenicity of the vector strains was analyzed in a rabbit model of intestinal colonization (8, 33). Oral immunization of rabbits was evaluated by measuring vibriocidal antibody titers, anti-CtxB IgG antibody titers, and anti-LPS antibody titers. As shown in Table 3, the high serum anti-*V. cholerae* antibody responses produced by the rabbits on days 14 and 21 postinoculation indicated that rabbits were successfully colonized by *V. cholerae*. Comparison of the serologic responses to the B subunit of cholera toxin following orogastric inoculation with the wild-type C6709 and Peru-10 (in which *ctxB* is expressed from *htpGp*) suggested that both the cholera toxin and heat shock promoters are active in vivo, eliciting comparable immunologic responses. High levels of biliary anti-CtxB IgA antibodies were detected following orogastric inoculation of rabbits with wild-type C6709 (*ctxP→ctxB*) and Peru-10 (*htpGp→ctxB*), suggesting that production of mucosal IgA may be a significant component of the immune response to these vaccine strains.

Serum IgG and bile IgA antibody responses to Slt-IB produced by *V. cholerae* vector strains. The immune response to the Slt-IB protein expressed by the vector strains following oral immunization was evaluated in a rabbit model (8, 33). Low serum IgG antibodies to Slt-IB were obtained when JRB11 or JRB12 was used as the inoculum. Peru-10 and JRB10 failed to provoke detectable anti-Slt-IB-specific IgG antibodies. However, all four strains produced biliary IgA responses specific for Slt-IB (Table 4).

No correlation was seen between the type of promoter controlling *slt*-IB and the degree of serum IgG response; neither Peru-10 (*irgP→slt*-IB) nor JRB10 (*irgP, htpGp→slt*-IB) evoked measurable serum IgG to Slt-IB, while immunization with JRB11 (*htpGp→slt*-IB) induced a low anti-Slt-IB IgG response. The absence of measurable serum anti-Slt-IB IgG in response to Peru-10 is consistent with the low levels of expression of Slt-IB from Peru-10 observed in vitro (Table 2). However, JRB10, which produced more Slt-IB in vitro than JRB11, did not induce a measurable serum anti-Slt-IB IgG response, while JRB11 did induce a small serum IgG response. In contrast, the number of promoters expressing *slt*-IB corresponded to the level of serum anti-Slt-IB IgG response observed. JRB12, containing two copies of the gene for *slt*-IB, evoked a larger serum IgG response than JRB10 or JRB11, each containing a single copy of *slt*-IB, consistent with the increased in vitro expression of Slt-IB in JRB12 compared with JRB10 and JRB11.

All four vector strains induced moderate biliary IgA responses, even in the absence of detectable serum IgG responses; no correlation was observed between the type of promoter expressing *slt*-IB in the various strains and the level of biliary anti-Slt-IB IgA response. In vitro production of Slt-IB did not predict in vivo anti-Slt-IB biliary IgA levels; JRB10 produced more Slt-IB in vitro than Peru-10 or JRB11 but induced the lowest levels of anti-Slt-IB biliary IgA. Immunization of rabbits with Peru-10, containing *htpGp→ctxB*, produced marked serum IgG and biliary IgA responses to CtxB (Table 3); in comparison, serum IgG and biliary IgA responses to Slt-IB, when *slt*-IB is also expressed from *htpGp*, as in JRB10, JRB11, and JRB12, were much lower.

DISCUSSION

The live, attenuated strains of *V. cholerae* described in this study have many of the characteristics required for effective oral bacterial vaccine vectors. Live vaccine strains should be safe and nonreactogenic; in this study, the parent strain, *V. cholerae* El Tor mutant Peru-2, lacks the virulence genes *ctxAB*, *zot*, *ace*, and *cep* and is presumed to be incapable of

TABLE 3. Antibody responses by ELISA to *V. cholerae* antigens following single orogastric inoculation of rabbits with 10^{10} organisms

Strain	Titer									
	Serum vibriocidal antibody			Serum anti-LPS IgG			Serum anti-CTX B subunit IgG			Bile anti-CTX B subunit IgA, day 21
	Day 0	Day 14	Day 21	Day 0	Day 14	Day 21	Day 0	Day 14	Day 21	
C6709	<25	800	3,200	<100	1,600	12,800	<10	40	640	12,800
Peru-10	<25	6,400	12,800	<100	1,600	6,400	<10	40	160	12,800
JRB10	<25	3,200	3,200	<100	1,600	25,600	ND[a]	ND	ND	ND
JRB11	<25	12,800	6,400	<100	3,200	25,600	ND	ND	ND	ND
JRB12	<25	12,800	12,800	<100	3,200	12,800	<10	<10	<10	<10

[a] ND, not done.

TABLE 4. Antibody responses by ELISA to Slt-IB following single orogastric inoculation of rabbits with 10^{10} organisms

Strain	Response[a]			
	Serum IgG			Biliary IgA, day 21
	Day 0	Day 14	Day 21	
C6709	2	2	2	10
Peru-10	2	2	2	160.0
JRB10	2	2	2	33.6
JRB11	2	4.0	3.4	226.3
JRB12	2	50.8	45.3	226.3

[a] Expressed as the geometric mean titer of two to four separate measurements for each sample. For purposes of calculating the geometric mean titer, measurements negative at all dilutions were assigned values of half the lowest dilution tested. A value of 2 reflects a consistently negative reading at the starting serum dilution of 1:4. A value of 10 reflects a consistently negative reading at the starting bile dilution of 1:20.

reacquiring the CTX genetic element by site-specific recombination because RS1 and the *attRS1* site are deleted (32, 41). In addition, a vector strain that delivers heterologous antigens should optimally have the genes encoding those antigens stably inserted into the host chromosome, rather than carried on a plasmid, to avoid the potential loss of these genes during bacterial replication. In this report, we introduce a rapid technique for performing in vivo marker exchange onto the *V. cholerae* chromosome. This method uses plasmid p6891MCS to insert markers of interest into the *V. cholerae lacZ* gene, allowing for detection of recombinants by simple color screening and antibiotic selection. It is widely applicable for placing any construct into a non-virulence-associated locus of the chromosome. Finally, live vectors should be capable of colonizing the host. Intestinal colonization of suckling mice and adult rabbits correlates with immunogenicity of live cholera vaccines in humans (33, 41). The deletion of the CTX genetic element and the insertion of *slt*-IB within *irgA* and *lacZ* did not substantially affect the ability of the strains to colonize experimental animals and to act as effective vectors, as demonstrated by the ability of these strains to colonize suckling mice and to stimulate high levels of serum and mucosal antibodies to *V. cholerae* antigens following oral immunization of rabbits. Taken together, the properties possessed by these strains indicate that the methods of strain construction described in this report will be very useful in the creation of live vaccine vectors.

We have chosen to use the nontoxic B subunit of Slt-I as a model vectored heterologous antigen. This antigen was selected because of the simple ELISAs for its detection, as well as the possible role that antibodies against the B subunit may play in protecting against severe shigellosis due to *S. dysenteriae* 1 and hemolytic uremic syndrome or hemorrhagic colitis due to Slt-producing *E. coli*. The immune response to Slt-I is primarily directed against the B subunit; antibodies directed against the B subunit or against synthetic peptides based on its sequence provide protective immunity against holotoxin (4, 12, 18, 19). All four vector strains induced moderate anti-Slt-IB biliary IgA responses, even in the absence of detectable serum IgG responses, suggesting that the vectors can colonize the small intestine and present Slt-IB to intestinal M cells in quantities adequate to evoke a mucosal IgA response. In this study, no correlation was observed between the type of promoter expressing *slt*-IB and the level of serum IgG or biliary IgA response, but the vector strain containing two copies of the gene for *slt*-IB evoked greater serum IgG responses than strains containing a single copy. However, comparison of the weak serum and biliary antibody responses to Slt-IB expressed from *htpGp* with the marked responses to CtxB expressed from the same promoter suggests that the antibody responses to Slt-IB evoked by the vector strains are not a result of poor promoter function of *htpGp* in vivo, but rather reflect the inability of Slt-IB to function as a strong immunogen in this delivery system. One possible explanation for this observation is inherent antigenic differences between the two subunits. This possibility is consistent with clinical observations indicating that Slt-I and Shiga toxin are poorly immunogenic in patients diagnosed with hemolytic uremic syndrome and *S. dysenteriae* 1 dysentery, respectively (17, 25). Alternatively, less Slt-IB than CtxB may be presented to the gut mucosa by this delivery system. Unlike CtxB, Slt-IB is not secreted from *V. cholerae* but remains in the periplasm (5) until released from the bacterium following cell lysis.

Because several bacterial virulence factors are produced in low-iron conditions, and in vivo-grown *V. cholerae* expresses proteins that may correspond to iron-regulated proteins (36), it has been hypothesized that bacteria sense low-iron conditions in the intestine. However, recent work by Camilli et al. (7) using in vivo expression technology suggested that the intestine is not a low-iron environment for *V. cholerae*. In the present study, rabbits orally immunized with Peru-10, expressing Slt-IB from the iron-regulated *irgA* promoter, did not develop systemic anti-Slt-IB antibodies but did develop a mild biliary anti-Slt-IB IgA response, indicating modest in vivo expression. Given the low level of antibody responses observed with all of the vector strains, however, it may be that high-level constitutive promoters or other in vivo-expressed promoters would be more effective for expressing heterologous antigens in this system. The *irgA* locus may function as a site on the *V. cholerae* chromosome where heterologous antigens may be inserted to attenuate virulence, with the antigens being expressed from a variety of other promoters. In addition, repeated oral doses of the vector strains may be needed to evoke higher levels of antibody responses. *Vibrio*-vectored antigens have been significantly boosted by a second oral immunization (17a). Experiments are planned to test whether a second oral dose of JRB12 will boost the anti-Slt-IB antibody response.

One advantage of oral vaccines over parenteral vaccines is that the direct administration of antigens appears to be the most efficient stimulus for development of secretory IgA (37); secretory IgA itself is sufficient to protect against intestinal disease from *V. cholerae* (45). We therefore were particularly interested in determining whether the *V. cholerae* vector strains would induce secretory IgA responses in orally immunized animals. The biliary anti-Slt-IB IgA response detected in rabbits immunized with the vectors suggests that protective immunity against holotoxin may be present in the intestine. Shiga-like toxin challenge studies of rabbits immunized with these constructs are under way to address this issue.

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