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Construction of a *Vibrio cholerae* Vaccine Candidate Using Transposon Delivery and FLP Recombinase-Mediated Excision

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Recent efforts to develop a vaccine against the diarrheal disease cholera have focused on the use of live attenuated strains of the causative organism, *Vibrio cholerae*. The Ogawa lipopolysaccharide phenotype is expressed by many epidemic strains, and motility defects reduce the risk of reactive diarrhea in vaccine recipients. We therefore converted a motile Inaba$^+$ vaccine candidate, Peru-2, to a nonmotile Ogawa$^+$ phenotype using a *mariner*-based transposon carrying *rfbT*, the gene required for expression of the Ogawa phenotype. Analysis of 22 nonmotile Peru-2 mutants showed that two were Ogawa$^+$, and both of these strains had insertions in the *flgE* gene. It was possible to convert these strains to antibiotic sensitivity by introducing a recombinase that acts on sites flanking the antibiotic marker on the transposon. The resulting strains are competent for colonization in infant mice and may therefore be suitable as vaccine candidates for use either independently or in a combination with strains of different biotypes and serotypes.

The gram-negative bacterium *Vibrio cholerae* is the causative agent of the diarrheal disease cholera. Infection typically begins with ingestion of *V. cholerae* in contaminated food or water. Upon colonization of the host, *V. cholerae* produces an A-B exotoxin (cholera toxin) that acts on the cells of the intestinal epithelium to induce a secretory diarrhea so severe that death can result within a few days (7, 20, 29). The disease is endemic in the Indian subcontinent and is estimated to affect millions of persons worldwide each year (19).

Efforts to combat cholera depend primarily on oral rehydration therapy, which is both simple and relatively inexpensive (5, 40). The importance of safe water supplies and adequate sanitation has also long been recognized (43, 51, 57). Nevertheless, as evidenced by the large number of cholera-related deaths reported annually, there are practical obstacles to applying any of these strategies consistently and effectively, particularly in underdeveloped areas of the world. This has made the need for a cholera vaccine increasingly urgent.

Killed whole-cell formulations, purified cholera toxin B subunit, and purified lipopolysaccharide (LPS) have been tested as vaccines, but none has demonstrated both efficacious and long-term protection against the disease (35). Based on evidence that development of long-lived protection against *V. cholerae* infection is strongly favored by clinical infection and presentation of vibrio immunogens to the mucosal immune system (24, 47), recent attempts to develop cholera vaccines have focused on the use of live, attenuated strains. Many of these strains are indeed immunogenic but also cause mild diarrhea in human volunteers (30, 34, 58). There were indications that colonization of the gut was required for immunogenicity but necessarily resulted in reactogenicity (60), and it seemed that this might constitute a considerable obstacle to the construction of acceptable vaccine strains. Fortunately, nonmotile vaccine candidates demonstrate both good immunogenicity and low reactogenicity (13, 33, 60), although the exact contribution of motility to *V. cholerae* infection is unknown.

Of the at least 151 recognized *V. cholerae* serogroups, only O1 and O139 serovars are thus far known to cause epidemic cholera. Strains of the O1 serogroups are divided into two biotypes, classical and El Tor (32, 53). Two of the six cholera pandemics since 1817 are known to have been caused by classical biotype strains, but the El Tor biotype is responsible for the current pandemic (4). The O139 serovar emerged in the Indian subcontinent in 1992 and was the first non-O1 serovar known to cause epidemic cholera (2, 49). O139 strains closely resemble O1 El Tor strains but possess a unique O antigen and are encapsulated (27, 64).

The vast majority of strains within the O1 serogroup display one of two serotypes that correspond to the expression patterns of certain LPS antigens. Inaba strains express the A and C antigens, while Ogawa strains express the A and B antigens and a small amount of the C antigen (41). The precise nature of the A, B, and C antigens is unknown. However, it has been shown that the *rfbT* gene determines the difference between the Ogawa and Inaba serotypes, in that the presence of *rfbT* is sufficient for Inaba-to-Ogawa serotype conversion (55).

The first *V. cholerae* isolates from the Latin American epidemic were identified as O1 Inaba strains, but Ogawa strains appeared within 8 months (59, 63) and 90% of Peruvian *V. cholerae* isolates were of the Ogawa serotype by 1995 (17, 62). Ogawa strains are prevalent in cholera-affected areas around the world (1, 10, 16, 22, 26, 31, 45, 48, 65), and since protective immunity against *V. cholerae* infection is provided in large part by anti-LPS antibodies (44, 56), the development of Ogawa vaccine strains may be highly advantageous. It has already been suggested that the most effective cholera vaccine might consist of a combination of strains of different biotypes and serotypes and that development of an El Tor Ogawa vaccine strain would be critical in such an approach (33, 61).

The vaccine candidate Peru-15 is a stably nonmotile, nontoxinogenic derivative of the Peruvian isolate C6709 (33). The genetic lesion resulting in nonmotility in Peru-15 has not been characterized at the molecular level. We therefore decided to construct a new Peru derivative by inserting *rfbT* into a motility

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
E. coli strains		
SM10 λ *pir*	*thi thr leu tonA lacY supE recA*::RP-4-Tc::Mu (λ *pir*)	42
SC625	TOP10 TnFC-*rfbT* mutant; Cm^r	This study
V. cholerae strains		
E7946	El Tor; Ogawa	
Peru-2	C6709 Δ*attRS1*; Inaba	60
Peru-15	C6709 Δ*attRS1 recA*::*htpG-ctxB*; spontaneously nonmotile; Inaba	33
NM3	Peru-2 *flgE*::TnFC-*rfbT* Fla^-; Ogawa	This study
NM11	Peru-2 *flgE*::TnFC-*rfbT* Fla^-; Ogawa	This study
SC631	NM3 ΔCm^r; Ogawa	This study
SC632	NM11 ΔCm^r; Ogawa	This study
Plasmids		
pBR322	Standard cloning vector; Ap^r	New England Biolabs
pCP20	FLP recombinase expression plasmid; pSC101 origin of replication; Ap^r	8
pCR2.1	Cloning vector; Ap^r	Invitrogen
pNEB193	Standard cloning vector; Ap^r	New England Biolabs
pSC121.1	*rfbT* gene from E7946 cloned into pCR2.1; Ap^r	This study
pSC138	TnFC-*rfbT*; Cm^r	This study
pSC141	Replicon fusion of pCP20 and pBR322; Ap^r Tc^r	This study

gene, thereby simultaneously producing an $Ogawa^+$ phenotype and a defined motility defect. Since it was unknown what level of *rfbT* expression would be necessary to convert a C6709 derivative to $Ogawa^+$, transposon delivery was selected as the method for introducing *rfbT* into the target strain. It was reasoned that because some bacterial motility genes are quite highly expressed, transposon delivery into a motility gene could place *rfbT* under the control of a sufficiently active promoter to result in an Ogawa phenotype. *mariner* transposons were particularly suited to this approach, since they have relatively low site specificity, increasing the chances of obtaining an insertion into a site favorable for expression of *rfbT*.

Since the presence of antibiotic markers is undesirable in vaccine candidates, the delivery transposon was engineered such that its chloramphenicol resistance (Cm^r) allele is flanked by directly repeated FRT sites. The yeast FLP recombinase catalyzes excision of sequences flanked by directly repeated FRT sites (14, 15), and the FRT-Cm^r cassette therefore permits the use of chloramphenicol selection to isolate transposon mutants and subsequent FLP-mediated excision to remove the Cm^r marker from the chromosome. The *rfbT* delivery transposon was used to obtain nonmotile mutants of Peru-2, a motile precursor of Peru-15 (A. Roberts, G. D. N. Pearson, and J. J. Mekalanos, Proc. 28th Joint Conf. U.S.-Japan Coop. Med. Sci. Program Cholera Relat. Diarrheal Dis., abstr., 1992). The nonmotile mutants were then screened for expression of Ogawa antigen. Two nonmotile, $Ogawa^+$ strains were isolated, and these were found to harbor independent insertions in the *flgE* gene. Expression of FLP recombinase in these strains resulted in loss of Cm^r without affecting the motility and Ogawa phenotypes. The Cm^s derivatives were aflagellar and competent for colonization of infant mice.

Although the *mariner*-FRT transposon delivery system was developed to create a nonmotile, $Ogawa^+$ C6709 derivative, this method should prove broadly useful. A similar system employing Tn*5* has been used to target DNA to the *Escherichia coli* chromosome (25), but use of a *mariner* transposon offers the advantages of extremely low site specificity and an exceptionally broad host range that includes both gram-positive and gram-negative bacteria (52). The *mariner*-FRT system should therefore facilitate the construction of vaccine strains for a variety of pathogenic bacteria, including those for which it has been difficult to implement allelic replacement or conventional transposon delivery strategies.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. All strains were stored at −75°C after addition of 80% (vol/vol) glycerol to cultures to a final concentration of 20% glycerol. All plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, Calif.).

Media and buffers. All of the buffers and media employed have been described previously. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml for *E. coli* and 1 μg/ml for *V. cholerae*; kanamycin, 30 μg/ml; streptomycin, 100 μg/ml; tetracycline, 12.5 μg/ml for *E. coli* and 1.25 μg/ml for *V. cholerae*.

Nucleic acid manipulations. All nucleic acid manipulations were accomplished in accordance with standard molecular biology techniques (3). Cloning of the *rfbT* PCR product was accomplished using the TOPO TA Cloning kit (Invitrogen, Carlsbad, Calif.) in accordance with the manufacturer's directions. Preliminary sequence data were obtained from The Institute of Genomic Research website at http://www.tigr.org.

Construction of pSC138. The 3.1-kb *Nae*I/*Not*I fragment of pBCMar-EnterpriseXN was ligated to the 2.2-kb *Eco*RV/*Not*I fragment of pBlue-R6K RP4 (28) to create pSC126. The β-lactamase gene from pBluescript KS(+) was then amplified by PCR and cloned into the *Not*I site of pSC126, producing pSC127.1. Next, oligonucleotide oSC53 (5′ TCTAGAGAATAGGAACTTCGGAATAGG AACTTCAGACCGGGGACTTATCAGCCAACCTGTTAG 3′) was used to amplify a 5.2-kb product from pSC127.1 and this product was treated with T4 polynucleotide kinase and self-ligated to create pSC130. A 5.2-kb fragment was similarly amplified from pSC127.1 using oligonucleotide oSC54 (5′ TCTAGAA AGTATAGGAACTTCAACGCGTAGTCTGGGACGTCGTATGGGTAAG ACCGGGGACTTATCAGCCAACCTGTTA 3′), kinased, and self-ligated to produce pSC132. The 2.7-kb *Xba*I fragment of pSC130 and the 2.5-kb *Xba*I fragment of pSC132 were then ligated together to produce pSC133. This plasmid carries a single FRT site flanked by *mariner* arms, with a hemagglutinin epitope at the 3′ end of the FRT site.

PCR amplification of pBCMar-conditional using oligonucleotides oSC55 (5′ CGGGATCCGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTAT AGGAACTTCGGCGCGCCTACCTGTGACGGAAGATCACT 3′) and oSC56 (5′ CGGGATCCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGG AATAGGAACTTCATTTAAATGGCGCGCCTTACGCCCCGCCCTGCCA CTC 3′) produced a chloramphenicol resistance allele flanked by directly repeated FRT sites, and this product was cloned into the *Sma*I site of pUC19 to create pSC136. Digestion of pSC136 with *Xba*I released a 932-bp fragment that was then cloned into pSC133 linearized by partial digestion with *Xba*I. The resulting plasmid (pSC137) carries a *mariner* transposon containing a chloramphenicol resistance allele flanked by directly repeated FRT sites. The 2.1-kb *Pst*I/*Sac*II fragment from pSC121.1 was subsequently cloned into the *Mlu*I site of

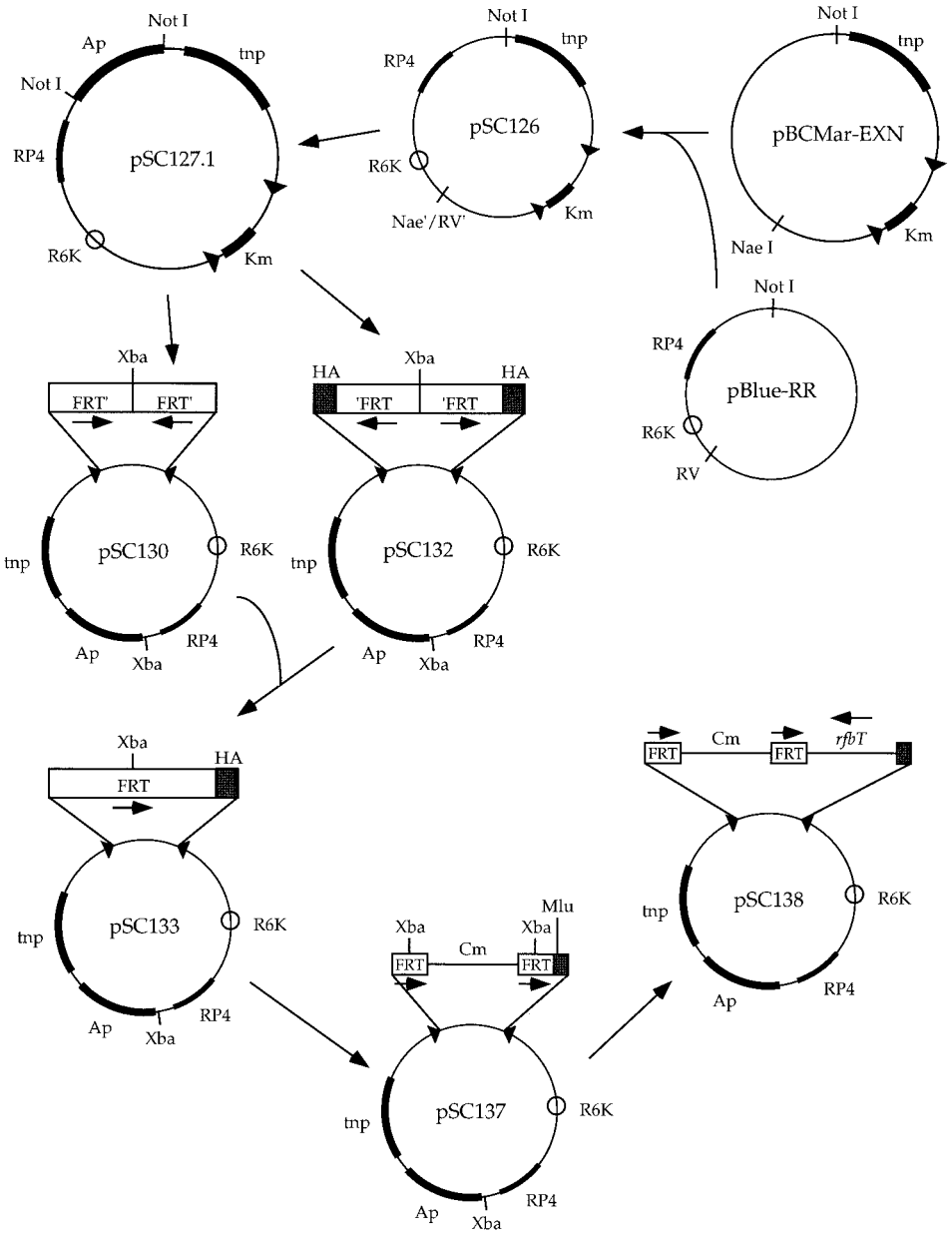

FIG. 1. Construction of pSC138. Abbreviations: Ap, ampicillin resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance; R6K, *ori*R6K conditional origin of replication; RP4, origin of transfer; tnp, transposase; HA, influenza virus hemagglutinin epitope.

pSC137 to create pSC138. The sequences of the FRT sites in pSC138 were verified.

Bacterial matings. *E. coli* strain SM10 λ *pir* (42) was used as the donor strain for all matings. Fresh cultures of donor and recipient strains were washed once in Luria broth (LB), mixed together on L agar, and incubated at 37°C for 4 to 6 h.

Slide agglutination assay. Bacterial cultures grown overnight at 37°C were mixed with an equal volume of *V. cholerae* anti-Ogawa or anti-Inaba typing serum (Difco, Inc., Detroit, Mich.) and scored visually for agglutination.

Electron microscopy. Each sample was prepared by floating a carbon type A grid (300-mesh copper; Ted Pella, Inc., Redding, Calif.) on a 50-μl drop of an overnight bacterial culture for 5 min with the Formvar surface facing the drop. The grid was then transferred to a 50-μl drop of 0.5% (wt/vol) phosphotungstic acid (pH 6.5) for 30 s. Samples were viewed with a JEOL JEM-1200EX electron microscope at 60 kV.

Induction of FLP-mediated recombination. Plasmids carrying the FLP recombinase gene (pCP20 and pSC141) were introduced into *E. coli* and *V. cholerae* strains by electroporation. *E. coli* was cultured overnight in LB containing chloramphenicol (20 μg/ml) and streptomycin (100 μg/ml). Cells from 0.5 ml of the culture were washed three times in 0.5 ml of sterile water and resuspended in 40 μl of sterile water prior to electroporation. For *V. cholerae* strains, overnight cultures were subcultured 1:1,000 in LB containing chloramphenicol (1 μg/ml) and streptomycin (100 μg/ml) and grown to mid-log phase at 37°C on a roller shaker. Cells from 1.5 ml of a mid-log-phase culture were washed three times in 1 ml of ice-cold 2 mM $CaCl_2$ and then resuspended in 40 μl of ice-cold 2 mM $CaCl_2$. Electroporation was carried out with a Bio-Rad Gene Pulser and Pulse Controller using cuvettes with a 0.1-cm (for *E. coli*) or a 0.2-cm (for *V. cholerae*) gap distance and the following settings: 1.8 kV, 25 mF, and 200 Ω (Bio-Rad Laboratories, Inc., Hercules, Calif.). Cells were allowed to recover at 30°C (for 1 h with agitation for *E. coli* and for 2 h without agitation for *V. cholerae*), plated on L agar containing ampicillin (100 μg/ml), and grown overnight at 30°C. A single isolate from each electroporation was then streaked on L agar containing streptomycin (100 μg/ml) and grown overnight at 37°C. Colonies from these restreaks were screened for chloramphenicol and ampicillin sensitivity by patching.

Infant mouse colonization assay. Colonization assays were done as described previously (9), with minor modifications. Strains SC631 and SC632 were grown

TABLE 2. Colonization data for SC631, SC632, and Peru-15

Strain[a] and mouse no.	Total no. of CFU in intestine
SC631	
1	5.0×10^4
2	8.0×10^3
3	3.6×10^5
4	6.0×10^4
5	8.7×10^4
6	3.0×10^5
7	6.4×10^4
SC632	
1	9.1×10^4
2	1.5×10^5
3	0
4	2.0×10^3
5	4.0×10^3
6	2.0×10^4
7	1.3×10^5
Peru-15	
1	2.0×10^5
2	6.3×10^4
3	0
4	4.3×10^7
5	0
6	4.4×10^4
7	2.4×10^7
8	2.4×10^8
9	0

[a] Input numbers of bacteria per mouse: SC631, 8.0×10^7 CFU; SC632, 3.8×10^6 CFU; Peru-15, 5.0×10^6 CFU.

overnight at 37°C in LB containing streptomycin (100 μg/ml). Bacteria were pelleted in a microcentrifuge, washed once in 1 volume of LB, resuspended in 1 volume of LB, and mixed with 10 μl of blue food dye per ml of bacterial suspension. For the Peru-15 colonization assay, lyophilized vaccine obtained from D. N. Taylor (U.S. Army Medical Research Institute for Infectious Diseases) was reconstituted with sterile water and 1 ml of this reconstituted vaccine was mixed with 10 μl of blue food dye. Each inoculum was administered perorally to 6-day-old CD1 mice (Charles River, Inc., Wilmington, Mass.) at a dose of 50 μl per mouse and plated at various dilutions on L agar containing streptomycin (100 μg/ml) to determine the number of CFU present in the inoculum. The small bowel of each mouse was recovered 20 to 24 h later, homogenized in 5 ml of LB, and plated at various dilutions on L agar containing streptomycin (100 μg/ml) to determine the number of CFU present.

RESULTS

Complementation of Peru-15 to Ogawa$^+$ by the *rfbT* allele of E7946. Earlier work demonstrated that the *rfbT* gene alone is sufficient to convert *V. cholerae* Inaba strains to Ogawa, and the transcriptional start site of *rfbT* has been mapped by primer extension (55). PCR primers were designed to include this start site and an additional 100 bp of the 5′ flanking sequence. After amplification of the *rfbT* gene from *V. cholerae* strain E7946 (El Tor, Ogawa), the product was cloned into pCR2.1 and the resulting plasmid, pSC121.1, was introduced into *V. cholerae* strain Peru-15 (33) by electroporation. The presence of pSC121.1 rendered Peru-15 agglutinable by anti-Ogawa typing serum but did not abolish the ability of the strain to be agglutinated by anti-Inaba typing serum.

Construction of a *mariner* transposon vector for delivery of *rfbT*. The construction of pSC138 is described in Fig. 1 and Materials and Methods. The *mariner* transposon on this plasmid (TnFC-*rfbT*) consists of a chloramphenicol resistance allele flanked by directly repeated FRT sites and the *rfbT* gene from pSC121.1. Although the *rfbT* gene is presumed to possess its own promoter, the gene is oriented such that its expression can be driven by a promoter adjacent to a transposon insertion. pSC138 also carries the gene encoding the *Himar1* transposase, an oriR6K origin of replication, and the RP4 origin of transfer.

Isolation and characterization of nonmotile Ogawa$^+$ Peru-2 transposon mutants. The motile *V. cholerae* strain Peru-2 (Roberts et al., Proc. 28th Joint Conf. U.S.-Japan Coop. Med. Sci. Program Cholera Relat. Diarrheal Dis.) was mutagenized with TnFC-*rfbT* by introducing pSC138 into Peru-2 by plate mating. The mating was then scraped into 50 ml of LB containing chloramphenicol (1 μg/ml) and streptomycin (150 μg/ml) and grown overnight at 37°C on a platform shaker. A 1-ml sample of a 1:1,000 dilution of this culture was added to 500 ml of 0.4% motility agar containing chloramphenicol (1 μg/ml) and streptomycin (100 μg/ml) and poured into petri dishes. After overnight growth at 37°C, putative nonmotile bacteria were picked and restabbed into motility agar for verification of their phenotype.

Two of 22 nonmotile isolates tested by slide agglutination were positive for expression of Ogawa antigen. Both isolates were ampicillin sensitive, indicating that the donor plasmid had not been retained. Electron microscopy (data not shown) demonstrated that NM3 and NM11 were aflagellar, and both were nonagglutinable by anti-Inaba typing serum. To determine the locations of the transposons in NM3 and NM11, the insertion junctions were cloned into pNEB193 and sequenced. The insertions were independent, but both occurred in a gene that is predicted to encode the flagellar hook protein FlgE. *rfbT* and *flgE* are oriented in the same direction in both strains.

FLP-mediated loss of chloramphenicol resistance. The temperature-sensitive plasmid pCP20 expresses the FLP recombinase under the control of the λ p_R promoter and the λ *c*I857 repressor (8), and the ability of pCP20 to mediate excision of the FRT-Cm^r-FRT cassette was confirmed as described in Materials and Methods using SC625, an *E. coli* strain harboring a TnFC-*rfbT* insertion. However, pSC101 derivatives replicate poorly, if at all, in *V. cholerae* (J.J.M., unpublished data) and all attempts to introduce pCP20 into NM3 and NM11 by electroporation were unsuccessful. Therefore, pCP20 and pBR322 were linearized with *Pst*I and ligated together to create a replicon fusion (pSC141) that could be maintained in NM3 and NM11 in the presence of ampicillin. Induction of FLP expression from pSC141 was successful and resulted in the isolation of chloramphenicol-sensitive derivatives of NM3 and NM11. Analysis of these strains (SC631 and SC632) demonstrated that they were Ogawa$^+$, nonmotile, aflagellar, and Inaba$^-$. Both were also ampicillin sensitive, indicating that pSC141 can be cured easily in the absence of ampicillin selection.

Colonization ability of SC631 and SC632. Since colonization may be critical for development of a protective immune response, SC631 and SC632 were examined for colonization ability using the suckling mouse model. The results (Table 2) demonstrate that both strains are competent for colonization of infant mice, although at slightly reduced levels compared to Peru-2 (6) and Peru-15.

To determine the stability of the Ogawa$^+$ LPS phenotype during infection, four postinfection isolates were chosen for each strain, with each isolate obtained from a different mouse. All eight isolates were agglutinable by anti-Ogawa typing serum but not by anti-Inaba typing serum. In addition, the motility phenotypes of 16 postinfection isolates of each strain were assessed by stabbing into motility agar and all postinfection isolates were found to be nonmotile. These data provide evidence that the *rfbT* insertions in SC631 and SC632 are stable during infection.

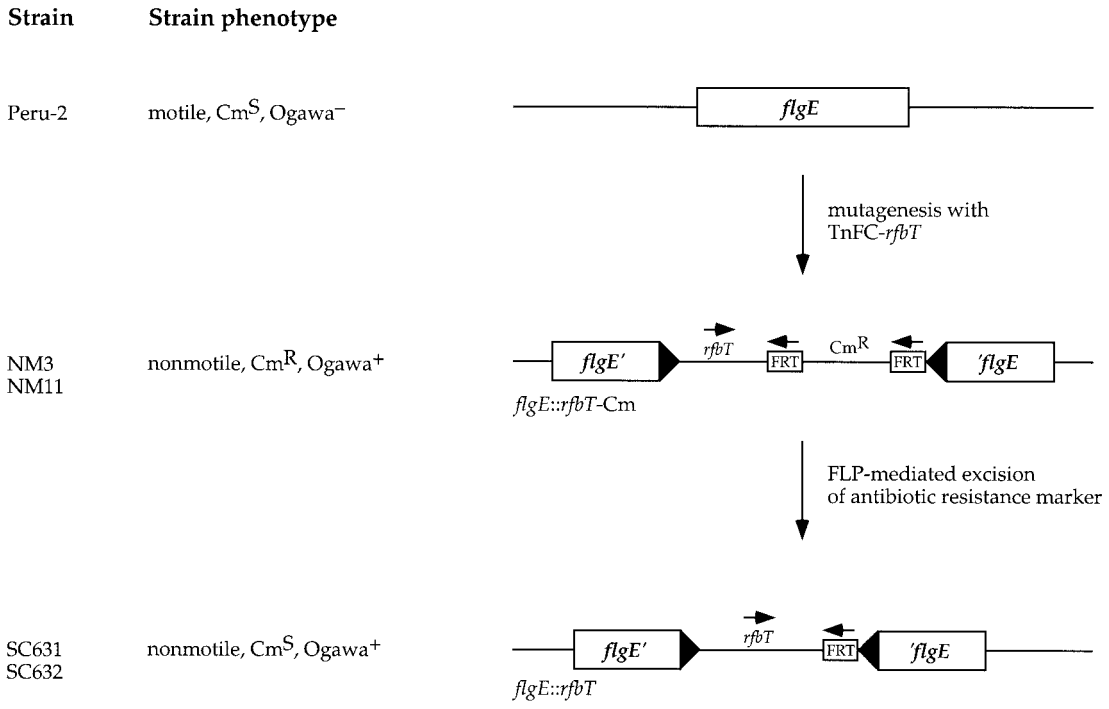

FIG. 2. Diagram of construction of nonmotile, Ogawa$^+$ strains using TnFC-*rfbT*.

DISCUSSION

This report describes the use of transposon delivery of the *rfbT* gene to obtain nonmotile, Ogawa$^+$ derivatives of *V. cholerae* strain Peru-2 (Fig. 2). The two strains thus isolated carried independent insertions in *flgE*, which is predicted to encode the flagellar hook protein. FLP-mediated recombination was subsequently used to remove the chloramphenicol resistance marker from the chromosomal insertions. These nonmotile, Ogawa$^+$, chloramphenicol-sensitive strains are competent for colonization as assessed in the suckling mouse model, and they may therefore be suitable as vaccine candidates either independently or in combination with other vaccine strains.

The *rfbT* allele complements Peru-15 to Ogawa when present on the multicopy plasmid pSC121.1, but it generally does not create the Ogawa phenotype in Peru-2 when inserted into the chromosome in single copy. This indicates that single-copy expression of *rfbT* from its own promoter is insufficient to convert a strain to Ogawa and that it was necessary to place *rfbT* under the control of a chromosomal promoter in order to achieve serotype conversion. Evidence supporting this hypothesis is provided by the fact that the two insertions leading to an Ogawa phenotype occurred such that the *rfbT* gene was oriented in the same direction as the gene into which it was inserted.

There are approximately 50 motility genes in *E. coli* (39) and a comparable number of proteins in the *V. cholerae* genome that are predicted to be involved in flagellar function (The Institute for Genomic Research, http://www.tigr.org). Although only 22 nonmotile mutants were screened for the Ogawa phenotype, the low site specificity of *mariner* transposons makes it somewhat surprising that the two Ogawa$^+$ isolates had independent insertions in *flgE*. The expression level of *flgE* relative to those of other *V. cholerae* motility genes is not known, but these data suggest that *flgE-rfbT* fusions result in appropriate levels of *rfbT* expression for conversion to Ogawa while fusions to other motility genes do not. This illustrates one advantage of using such a promoter trap strategy for heterologous gene expression. So long as there is a relatively simple assay for expression of the introduced gene, the empirical approach of generating a bank of transposon insertions and subsequently screening for strains displaying the desired traits may, in fact, be more effective than attempting to decide a priori which promoters or insertion sites might result in optimal levels of gene expression. It should, in certain instances, be possible to enrich or even select for strains that demonstrate the phenotype of interest.

The *mariner*-FRT system should be particularly useful for the construction of vaccine strains expressing specific antigens. FLP-mediated removal of antibiotic markers is simple and effective, and *mariner*-derived delivery vectors are especially attractive because of their low site specificity and broad host range. They are active not only in gram-positive and gram-negative bacteria but also in several eukaryotic organisms (18, 21, 36–38, 50, 52, 54, 66). This promiscuity could provide huge advantages in bacteria where there are no existing transposition systems (e.g., *Borrelia* spp.) or where current transposition systems demonstrate high site specificity (e.g., mycobacteria and gram-positive organisms).

The vaccine potential of the aflagellar, Ogawa$^+$ strains SC631 and SC632 remains to be determined. Peru-2 itself has not been tested for efficacy as a cholera vaccine, but several of its derivatives (Peru-3, -14, and -15) have been shown to elicit strong and, in some cases, protective immune responses against *V. cholerae* in human subjects (33, 60). Since Peru-14 and Peru-15 are motility deficient and exhibit lower reactogenicity than motile strains, it seems not unreasonable to expect that nonmotile strains SC631 and SC632 will also demonstrate low reactogenicity. One important advantage of pursuing vaccine development with SC631 and SC632 is that Peru-14 and Peru-15 were isolated as spontaneous nonmotile mutants, and

the molecular basis of their nonmotility is unknown. The fact that Peru-14 shows reversion to motility upon passaging (33) emphasizes the need for vaccine strains with defined motility defects.

Antibacterial immunity is thought to play the dominant role in protection against cholera, but the importance of antitoxic immunity was clearly demonstrated in a field trial in Bangladesh involving 89,000 persons. Vaccination with an oral B-subunit whole-cell vaccine provided better protection against cholera than oral whole-cell vaccine alone, although the increased efficacy of the B-subunit whole-cell preparation was evident only in the first 8 to 12 months after immunization (11, 12, 23). In order to promote a good immune response against cholera toxin, most of the Peru vaccine strains contain the *ctxB* gene under the control of the in vivo-inducible *htpG* promoter inserted into the *recA* locus (46, 52). It should be relatively simple to introduce the *recA*::*htpG-ctxB* mutation into SC631 and SC632, and this work is already under way. Once this is accomplished, it will be of great interest to determine the ability of those strains to stimulate long-lived immunity to cholera in human volunteers.

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