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Construction and Evaluation of a Safe, Live, Oral *Vibrio cholerae* Vaccine Candidate, IEM108

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IEM101, a *Vibrio cholerae* O1 El Tor Ogawa strain naturally deficient in CTXΦ, was previously selected as a live cholera vaccine candidate. To make a better and safer vaccine that can induce protective immunity against both the bacteria and cholera toxin (CT), a new vaccine candidate, IEM108, was constructed by introducing a *ctxB* gene and an El Tor-derived *rstR* gene into IEM101. The *ctxB* gene codes for the protective antigen CTB subunit, and the *rstR* gene mediates phage immunity. The stable expression of the two genes was managed by a chromosome-plasmid lethal balanced system based on the housekeeping gene *thyA*. Immunization studies indicate that IEM108 generates good immune responses against both the bacteria and CT. After a single-dose intraintestinal vaccination with 10^9 CFU of IEM108, both anti-CTB immunoglobulin G and vibriocidal antibodies were detected in the immunized-rabbit sera. However, only vibriocidal antibodies are detected in rabbits immunized with IEM101. In addition, IEM108 but not IEM101 conferred full protection against the challenges of four wild-type toxigenic strains of *V. cholerae* O1 and 4 μg of CT protein in a rabbit model. By introducing the *rstR* gene, the frequency of conjugative transfer of a recombinant El Tor-derived RS2 suicidal plasmid to IEM108 was decreased 100-fold compared to that for IEM101. This indicated that the El Tor-derived *rstR* cloned in IEM108 was fully functional and could effectively inhibit the El Tor-derived CTXΦ from infecting IEM108. Our results demonstrate that IEM108 is an efficient and safe live oral cholera vaccine candidate that induces antibacterial and antitoxic immunity and CTXΦ phage immunity.

Cholera is a severe secretory diarrheal disease caused by toxigenic *Vibrio cholerae* (7). *V. cholerae* is classified into about 200 serogroups on the basis of its somatic antigens. However, only serogroups O1 and O139 have been implicated in epidemics. Serogroup O1 can be further distinguished into two biotypes, classical and El Tor. The classical biotype was responsible for the first six pandemics, and the El Tor biotype was responsible for the seventh pandemic, which began in 1961 and is ongoing. Aside from O1, the new cholera pathogen *V. cholerae* O139 has caused epidemics in Southeast Asia since 1992. But, at present the El Tor biotype is still the main pathogen causing epidemics and isolated cases.

The most important virulence properties of *V. cholerae* are the production of cholera toxin (CT) and the ability to adhere to and colonize the small intestine of the host (18). CT is an enterotoxin consisting of one A (CTA) and five B (CTB) subunits encoded by *ctxA* and *ctxB*, respectively. The 11.6-kDa CTB is a nontoxic protective antigen responsible for mediating toxin binding to the GM1 ganglioside receptor on host intestinal epithelial cells. The 28-kDa CTA activates the cellular adenylate cyclase and accelerates the secretion of chloride and bicarbonate from the mucosal cells to the intestinal lumen (8).

Vaccination against cholera is a powerful and feasible disease prevention strategy, because recovery from infection results in long-term protective immunity (11, 27). Many parenteral cholera vaccines have been developed, including killed-whole-cell vaccines with or without various adjuvants and a purified lipopolysaccharide vaccine. Because of their poor immunogenicity and high reactogenicity and the mucosal nature of a *V. cholerae* infection, attempts to develop oral formulations that stimulate the mucosal immune system began in the 1980s (22). Generally, two strategies are being used to develop oral vaccines against cholera. One is based on a combination of killed whole *V. chlolerae* cells with CTB (17, 19, 22). The other is based on live, attenuated, CT-defective *V. cholerae* strains, thereby mimicking natural infection (11, 17, 19). Field trials showed that a vaccine consisting of whole cells plus a B subunit produced limited protection in children younger than 5 years. It also required multiple doses because of noncolonization in the human intestine (22, 27).

Compared to the killed-whole-cell vaccine, a live oral attenuated vaccine offers great promise for preventing cholera because a single dose could cause active colonization, elicit high-titer serum vibriocidal antibodies, and stimulate mucosal immunity that is able to block bacterial adherence, kill the bacteria, and neutralize the toxin, thus providing excellent protection (4, 26, 27). Several engineered live attenuated oral vaccine candidates have been developed, including CVD101,

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TABLE 1. Bacterial strains used in this study

Bacterial strain	Description	Source or reference
O395	*V. cholerae* O1, classical, Ogawa, CTXΦ$^+$	Patient
1119	*V. cholerae* O1, classical, Inaba, CTXΦ$^+$	Patient
Wujiang-2	*V. cholerae* O1, El Tor, Inaba, CTXΦ$^+$	Patient
Bin-43	*V. cholerae* O1, El Tor, Ogawa, CTXΦ$^+$	Patient
IEM101	*V. cholerae* O1, vaccine candidate strain, Ogawa, CTXΦ$^-$	16
IEM101-T	*V. cholerae* O1, derived from IEM101, Δ*thyA*	This study
PL101	*E. coli*, derived from *E. coli* DH5α, Δ*thyA*	30
SM10λ*pir*	*E. coli thi thr leu tonA lacY supE recA*::*RP4-2-TC*::Mu (*pirR6K*) Km^r	25

CVD103, CVD103-HgR, CVD111, Peru-14, and Peru-15 (9, 11, 13, 19, 27), and some have been used in clinical trials.

However, the discovery of *ctxAB* as part of the lysogenic phage CTXΦ genome showed that CTXΦ may transfer this enterotoxin gene from a toxigenic strain to a nontoxigenic strain (29) and thus raised safety concerns over the use of those genetically engineered live oral vaccines (12). Live vaccine strains may potentially regain virulence by acquiring the enterotoxin gene horizontally from toxigenic strains in the host intestine or in the environment, especially when they are used in epidemic areas where wild toxigenic strains may exist. So construction of a protective and safe live attenuated vaccine that is immune to CTXΦ infection is a problem to be solved.

The CTXΦ genome may be partitioned into the core region, which carries *ctxAB* and the genes required for virion morphogenesis, and the RS2 region, which carries *rstA* and *rstB*, which are required for replication and integration of the genome, and a repressor gene, *rstR* (28). Based on *rstR* sequences and their bacterial hosts, CTXΦ has several alleles (5, 12). Intraintestinal CTXΦ transduction assays have shown that the El Tor lysogens are immune to infection with El Tor-derived CTXΦ (CTX$^{ET\Phi}$), whereas the classical lysogen strains are not (12). This kind of CTXΦ immunity is biotype specific and is mediated by its repressor, RstR, through repressing expression of *rstA* and *rstB* (12). Therefore, introduction of a stably maintained *rstR* gene into a live *V. cholerae* vaccine strain will lower the possibility of vaccine reversion to toxigenicity due to CTXΦ infection and will thereby make it safer (12).

IEM101 (16, 17) is an ideal natural vaccine candidate strain selected in our laboratory previously. It is safe, protective, and immunogenic in rabbit models and human volunteers. To prevent it from regaining the *ctxAB* gene and bestow on it antitoxin immunity, an El Tor *rstR* gene and a *ctxB* gene were introduced into IEM101 by using a chromosome-plasmid lethal balanced system based on the housekeeping gene *thyA*. Thus a new vaccine candidate, IEM108, was constructed and evaluated for its immunogenicity and protective immunity against challenges in a rabbit model.

MATERIALS AND METHODS

***V. cholerae* O1 strains.** The bacterial strains used in this study are listed in Table 1. IEM101 (16), a live cholera vaccine candidate strain of the El Tor biotype, is naturally CTXΦ negative but has toxin-coregulated pilus (TCP) pili. Classical strains O395 (Ogawa) and 1119 (Inaba), as well as El Tor strains Wujiang-2 (Inaba) and Bin-43 (Ogawa), were used as the challenge strains in challenge and protection studies with rabbit models. All *V. cholerae* strains were propagated in Luria-Bertani (LB) broth or on gentamicin agar (GTA; peptone [10 g/liter], beef extract [3.0 g/liter], NaCl [5.0 g/liter], sucrose [10.0 g/liter], sodium citrate [10.0 g/liter], sodium sulfite [3.0 g/liter], agar [15 g/liter], gentamicin [500 μg/liter], polymyxin B [3,000 μg/liter]; pH 8.4 ± 0.1).

IEM101-T is a thymidine-auxotrophic strain derived from IEM101. It was previously constructed in our laboratory and has the central one-third of the thymidylate synthase gene (*thyA*) deleted from its genome. IEM101-T can grow in LB broth only with the addition of thymidine (50 μg/ml). However, when IEM101-T was transformed with the plasmid pXXB106 (30) (Table 2) carrying the *Escherichia coli*-derived *thyA* to complement its chromosomal *thyA* deletion, the transformed bacteria could be grown in LB broth without thymidine. This *thyA*-based chromosome-plasmid lethal balanced system was used to construct IEM108 by introducing plasmid pUTBL3 (Table 2) into IEM101-T.

***E. coli* strains used in molecular cloning and conjugation.** The *E. coli* strains used in this study are listed in Table 1. PL101 (30), a spontaneous *E. coli* DH5α *thyA* mutant, was used as the recipient of plasmid pUTBL3 containing *ctxB*, *rstR*, *E. coli*-derived *thyA*, and truncated *bla*. PL101 was grown in CBT medium (M9 minimal medium containing 0.5% glucose, 0.5% casein hydrolysate, and 0.1 μg of VitB/ml) supplemented with trimethoprim (15 μg/ml) and thymidine (50 μg/ml). For conjugation, SM10λ*pir* (25) was used as the recipient strain of recombinant suicide plasmids pKRSn and pKRSe (Table 2). The *E. coli* strain JM109 was used for transformation in general molecular cloning.

Genetic constructs. The plasmid constructs involved in genetic cloning and conjugation are listed in Table 2.

Construction of the candidate IEM108. The 1.15-kb *Xba*I fragment containing the upstream regulatory and coding regions of *ctxB* was recovered from pBR (a pUC19-derived plasmid carrying *ctxB* and *rstR*, constructed in our laboratory before) and cloned into the *Xba*I site of pXXB106, containing *E. coli*-derived *thyA* (30), resulting in two new constructs, pUTBL1-5 and pUTB1-6. *ctxB* and *thyA* have the same transcriptional direction in pUTBL1-5 and opposite directions in pUTBL1-6 (Fig. 1).

The *rstR* gene and its upstream sequence were amplified from El Tor strain Bin-43 with primers PrstR1 (CCGAATTCACTCACCTTGTATTCG) and PrstR2 (CGGAATTCTCGACATCAAATGGCATG). The amplified fragment was then cloned into the *Eco*RI site of pUTBL1-5, yielding new construct pUTBL2. Subsequently, an 0.8-kb *Pvu*I fragment of the *bla* gene in pUTBL2 was deleted to generate pUTBL3. pUTBL3 was then electroporated into IEM101-T to construct IEM108.

Conjugation. Plasmid pCT5A11 (8) contains a 15-kb *Hin*dIII fragment of El Tor strain 1621 including the CTX$^{ET\Phi}$ genome and its flanking sequence. After digestion with *Pst*I, the plasmid was blunt ended with T4 DNA polymerase and further digested with *Sac*I. The 3.6-kb fragment containing the RS2 sequence was gel purified and ligated into the suicide vector pKTN701 (pKTN701 was digested with *Xba*I, blunt ended with the Klenow fragment of DNA polymerase

TABLE 2. Plasmid constructs involved in this study

Plasmid	Description	Source or reference
pXXB106	DH5α-derived *thyA* cloned into pUC19, Amp^r	30
pKTN701	Suicide plasmid vector, *mobRP4 oriR6K* Cm^r	Gift from Jianguo Xu, our institute
pUTBL1-5	Derived from pXXB106; *ctxB* was cloned in the same transcriptional orientation as *thyA*	This study
pUTBL1-6	Derived from pXXB106; *ctxB* was cloned in the opposite transcriptional orientation to *thyA*	This study
pUTBL2	Derived from pUTBL1-5; El Tor-derived *rstR* was inserted upstream of the *thyA* gene and in the same transcriptional orientation	This study
pUTBL3	Derived from pUTBL2; the *bla* gene containing the *Pvu*I fragment was deleted	This study
pCT5A11	Containing El Tor-derived CTXΦ genome; Amp^r	8
pKRSn	pKTN701 containing RS2 of classical biotype-derived CTXΦ	This study
pKRSe	pKTN701 containing RS2 of El Tor-derived CTXΦ	This study

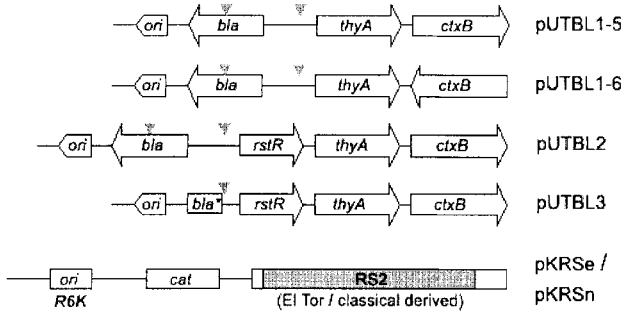

FIG. 1. Linear diagrams of plasmid constructs. Open boxes, gene segments; arrows, gene orientations; solid box, RS2 region, including *rstR*, *rstA*, and *rstB* genes from *V. cholerae* O1 El Tor strain in plasmid pKRSe or from a classical strain in pKRSn; arrowheads, *Pvu*I sites.

I, and then digested with *Sac*I) to generate plasmid pKRSe, which contains RS derived from the $CTX^{ET\Phi}$ genome. Plasmid pKRSn is also pKTN701 based and contains the RS sequence derived from the $CTX^{class\Phi}$ genome that was constructed in our previous work. These two recombinant suicide plasmids were used as a model to evaluate CTXΦ immunity.

SM10 carrying pKRSe or pKRSn was used as the donor strain to mate with IEM108 and IEM101, respectively. Mating mixtures were plated on the 0.22-μm-pore-size filter membrane stuck on the surface of LB agar and then cultured at 37°C for 6 h. The bacteria were washed and diluted with phosphate-buffered saline (PBS) and then plated on GTA selective media supplemented with 15 μg of chloramphenicol/ml to enumerate the number of transconjugants and on GTA without chloramphenicol to determine the total number of recipient strains. Transconjugant frequency is calculated as the ratio of transconjugants among recipients, as determined from the ratio of CFU of Cm^r Get^r colonies to CFU of Get^r colonies.

GM1-ELISA. A GM1–enzyme-linked immunosorbent assay (GM1-ELISA) was used to determine the expression of CTB in pUTBL1-5 and pUTB1-6 and anti-CTB titers in sera of immunized rabbits. A 96-well plate was coated with 2 μg of GM1(Sigma)/ml (100 μl/well) overnight at 4°C. Wells were washed three times with PBS plus 0.05% Tween 20 (PBT) and blocked with 1% bovine serum albumin (300 μl/well) in PBS for 1 h at 37°C. One hundred microliters of supernatant of JM109 cell lysate transformed with pUTBL1-5 and pUTB1-6 was added to each well, and the plate was incubated at 37°C for 1 h and washed again with PBT. The plate was then incubated with 100 μl of a 1:2,000 dilution of a polyclonal rabbit anti-CT antiserum/well for 1 h at 37°C, washed with PBT, and incubated for 1 h at 37°C with 100 μl of a 1:1,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG). The plate was subsequently washed with PBT. HRP-bound antibodies were visualized by adding *o*-phenylenediamine (Sigma) as the substrate. After 15 to 30 min the reaction was blocked by the addition of H_2SO_4 at a final concentration of 1 M and the absorption was read at 492 nm. Positive results were determined if the ratio of the sample to the control was ≥2. For the control we added supernatant of JM109 cell lysate.

The titer of the serum anti-CTB IgG antibody for the immunized rabbits was measured by GM1-ELISA as described above. Briefly, each well was coated with 100 μl of GM1 (2 μg/ml), saturated, and washed. Then 100 μl of CT (1 μg/ml) was added to each well, and the plates were incubated for 1 h at 37°C and washed again. The 1:5-diluted test sera (100 μl) were added to 100 μl of PBS in the first wells and then doubly diluted serially to 1:10,240. Plates were incubated and washed, and HRP-conjugated goat anti-rabbit IgG was added as described above. The preimmune sera were used as the negative control, and a 1:2,000 dilution of a polyclonal rabbit anti-CT antiserum was used as the positive control. The reciprocal titer is defined as the highest dilution of serum that gave an A_{492} ratio of sample to negative of ≥2.0.

Serum vibriocidal antibody assay. Serum vibriocidal antibody titers were measured in a microassay using 96-well plates. The immunized rabbit sera were inactivated at 56°C for 30 min and diluted 1:5 with PBS before use. The prediluted rabbit sera were added into the first well and then serially diluted threefold in PBS. PBS was added to the last well as a negative control. The plates were incubated for 30 min at 37°C with 25 μl of a solution containing 10^2 CFU of *V. cholerae* Bin-43/ml of culture and 20% guinea pig serum as a complement source in PBS. One hundred fifty microliters of 0.01% 2,3,5-trihenyltetrazolium chloride in LB broth was added to each well, and the plates were further incubated for 4 to 6 h at 37°C until the negative-control wells showed a color change. The reciprocal vibriocidal titer is defined as the highest dilution of serum that completely inhibits growth of Bin-43, i.e., no color change.

Rabbit immunization. Eight adult New Zealand White rabbits (2 to 2.5 kg) were divided into naive, IEM101, and IEM108 groups. The naive group consisted of two rabbits that were not immunized. Each immunization group had three rabbits. After fasting for 24 h, the rabbits in both immunization groups were anesthetized with ether. After the abdominal skin was sterilized with an iodine tincture and alcohol, the abdominal cavity was opened by vertical incision (under sterile conditions). General exploration was performed to find the ileocecal region. This region was ligated to the inner wall of the abdomen. Then 10^9 CFU of vaccine strain IEM101 or IEM108 were injected into the proximal ileum. Finally, the abdominal cavity was closed. The ligature that tied the ileocecal region to the abdominal wall was removed 2 h later, and the rabbits were given water and feed for 28 days. One rabbit of the IEM101 group died after the operation, probably because of heavy anesthesia. Serum samples were collected from the immunized rabbits prior to the immunization and on days 6, 10, 14, 21, and 28 after the vaccination. The serum titers for the anti-CT antibody and vibriocidal antibody were measured as described above.

Rabbit ileal loop assay and protection model. To evaluate the protection efficacy in vivo, the immunized rabbits were challenged with pure CT and four virulent *V. cholerae* strains (395, 119, Wujiang-2, and Bin-43) of different serotypes and biotypes (Table 1) 28 days after the single-dose immunization. Rabbits were anesthetized and their abdomens were opened as described above. Their intestines were tied into 4- to 5-cm-long loops, and then 10^5 to 10^8 CFU of challenge strains or 1, 2, 3, or 4 μg of pure CT was injected into each loop. Normal saline was used as negative control. At 16 to 18 h postchallenge, the rabbits were sacrificed and the accumulated fluid from each loop was collected and measured. The ratio of the volume of accumulated fluid (milliliters) to the length of the loop (centimeters) was calculated for each loop in the challenged rabbits.

RESULTS

Construction of IEM108 based on the *thyA* plasmid-chromosome lethal balanced system. In our previous work, we destroyed the *thyA* gene of IEM101 by deleting the central one-third of the gene, thus obtaining the thymidine-auxotrophic *V. cholerae* strain, IEM101-T. That study (30) showed that *E. coli*-derived *thyA* can complement the *thyA* function of *V. cholerae* and that plasmid pXXB106 containing *E. coli*-derived *thyA* can stably be maintained in a spontaneous *V. cholerae* Δ*thyA* mutant. When transformed with the plasmid pXXB106, IEM101-T grew well in LB medium. Therefore, we cloned the *ctxB* gene into pXXB106. To test if this cloned *ctxB* affects the expression of *thyA* because of its highly efficient promoter, plasmids pUTBL1-5 and pUTBL1-6, which have different insertion directions of *ctxB*, were electroporated into IEM101-T. We then assessed *V. cholerae* growth on LB media without thymidine. Comparisons of in vitro growth kinetics of IEM101 and IEM101-T (containing pUTBL1-5 and pUTBL1-6, respectively) showed a significantly reduced growth rate and an extended log phase for IEM101-T containing pUTBL1-6, whereas IEM101-T containing pUTBL1-5 and IEM101 had similar growth rates (Fig. 2). This observation may indicate that the reverse insertion of *ctxB* in pUTBL1-6 influenced the expression of *thyA* and therefore was responsible for the reduced levels of growth. Based on the different growth rates, we chose pUTBL1-5 for the cloning of *rstR*.

The fragment containing the *rstR* gene and its upstream sequence of the El Tor strain Bin-43 was amplified and cloned into the *Eco*RI site of pUTBL1-5 to generate pUTBL2. Thus, the cloned *rstR* gene is derived from the El Tor-derived $CTX^{ET\Phi}$ genome. In accordance with the proviso that an antibiotic resistance gene cannot appear in a recombinant plasmid which is introduced into the vaccine candidate, the *bla* in

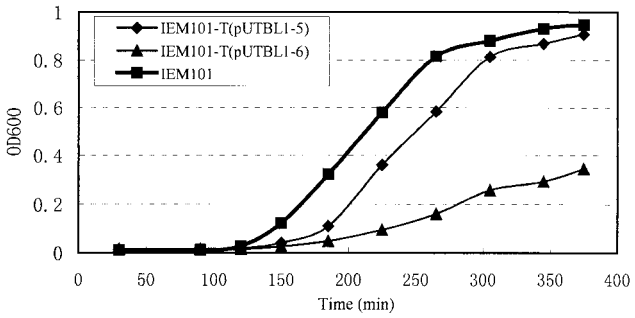

FIG. 2. Comparison of the growth curves of IEM101, IEM101-T containing pUTBL1-5, and IEM101-T containing pUTBL1-6. Single clones of IEM101, IEM101-T containing pUTBL1-5, or pUTBL1-6 were grown in LB broth at 37°C to exponential phase, and the optical density at 600 nm (OD_{600}) of each culture was adjusted to 0.3. Then 300 μl of each was inoculated into 30 ml of fresh LB broth in 100-ml flasks and grown aerobically at 37°C. The OD_{600} of each culture was detected at different times during growth.

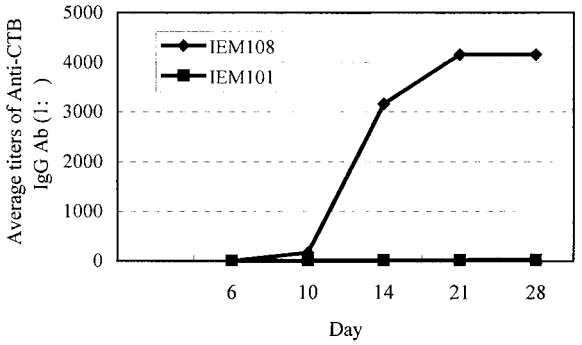

FIG. 3. Average titers of serum anti-CTB IgG antibodies after immunization with IEM101 and IEM108.

pUTBL2 was deactivated by deleting a 0.8-kb *Pvu*I fragment to generate pUTBL3. Therefore, pUTBL3 contains *ctxB*, *rstR*, *E. coli*-derived *thyA*, and the truncated *bla* gene. pUTBL3 was electroporated into IEM101-T to construct IEM108. pUTBL3 was maintained in IEM108 grown on LB agar plates without the need for antibiotic selection pressure.

The immunity of IEM108 against phage CTX$^{ET\Phi}$ infection and the ability to regain toxicity. Our previous studies have shown that the recombinant suicide plasmid containing RS2 could integrate into the chromosome of IEM101. In addition, this recombinant plasmid could replicate in its plasmid form (10). To test the ability of IEM108 to resist CTXΦ infection from both classical and El Tor sources and to test the cross-immunity induced by *rstR* against CTXΦ derived from different biotypes, the two SM10 donor strains, one containing pKRSn and one containing pKRSe, were used as a conjugation model. pKRSn contains the classical biotype-derived RS2 region, while pKRSe contains the El Tor-derived RS2 region. The average conjugation frequencies between recipient strains and donor strains are shown in Table 3. There was a 100-fold reduction in frequency of conjugation to the El Tor-derived RS2 region in IEM108 compared with the corresponding frequency for IEM101. However, when IEM101 and IEM108 mated with donor strain SM10 (containing pKRSn), the reduction of conjugation frequency for IEM108 was about ninefold less than that for IEM101 (Table 3).

TABLE 3. Conjugation of pKRSn and pKRSe from *E. coli* into *V. cholerae* IEM101 and IEM108[a]

Recipient strain	Donor strain	Mean conjugation frequency[b]
IEM101	SM10λ*pir*(pKRSn)	9.04×10^{-5}
	SM10λ*pir*(pKRSe)	1.26×10^{-5}
IEM108	SM10λ*pir*(pKRSn)	1.03×10^{-5}
	SM10λ*pir*(pKRSe)	1.03×10^{-7}

[a] Mating mixtures were plated on a filter film stuck on top of LB agar at 37°C for 6 h. Bacteria were washed down and diluted with PBS and then plated on GTA selective media supplemented with 15 μg of chloramphenicol/ml to enumerate the numbers of transconjugants. Bacteria were also plated on GTA without chloramphenicol to determine the total numbers of recipient strains.

[b] Mean fraction of transconjugants among recipients as determined from the ratio of CFU of Cm^r Get^r to Get^r bacteria.

The immunogenicity of IEM108 in (immunized) rabbits. Antibody analysis results for immunized rabbits showed that none of the rabbits produced anti-CTB antibodies and vibriocidal antibodies in their preimmune serum. After immunization, specific anti-CTB antibodies were detected in rabbit sera immunized with IEM108. They became detectable on day 10 after vaccination, and peak titer (1:4,155, on average) and plateau were reached on day 21 (Fig. 3). No anti-CTB antibody was detected in IEM101-immunized-rabbit sera. The vibriocidal antibody responses in sera from rabbits immunized with IEM101 and IEM108 were found to be very similar (Fig. 4). They became detectable on day 6 postimmunization and reached their peak on day 14. However, we found that the antibody titer for the IEM108 group is not only much higher than that for the IEM101 group but also decreases more slowly and thus lasts a longer time.

Protection against the challenges of toxigenic *V. cholerae* in vaccinated rabbits. Fluid accumulations of different rabbit groups are shown in Fig. 5 and 6. In the naive-animal group, all the loops had significant amounts of fluid accumulation after being challenged with either CT or bacteria. Furthermore, the higher the challenge dose, the more fluid was accumulated. In the group of IEM101-vaccinated animals, no significant fluid accumulation was detected in the loops subjected to a lower dose (10^5 to 10^7 CFU) of the bacterial challenge. However, when the challenge dose increased to 10^8 CFU, the loops in rabbits challenged with the classical strain 1119 and the El Tor

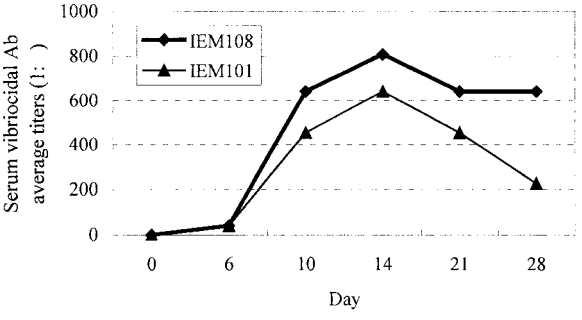

FIG. 4. Average titers of serum vibriocidal antibodies after immunization with IEM101 and IEM108.

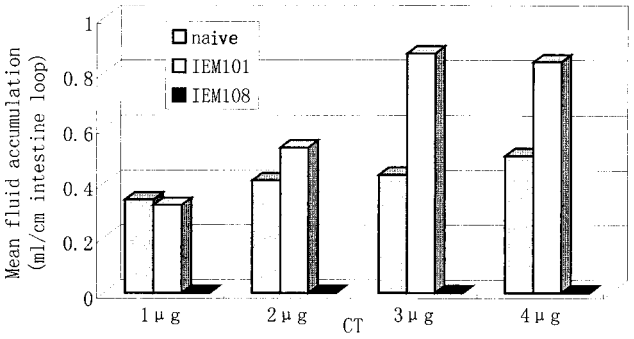

FIG. 5. Average fluid accumulations in the rabbit ileal loops after challenge with pure CT.

strain Wujiang-2 had average levels of fluid accumulation of 0.35 and 0.86 ml/cm of loop, respectively. All loops challenged with different doses of CT had very significant amounts of fluid accumulation: about 0.32, 0.53, 0.87, and 0.84 ml per cm of intestinal loop for 1, 2, 3, and 4 μg of CT, respectively. We detected no fluid accumulation in the group of IEM108-immunized rabbits after the challenge with either CT or bacteria, which indicates that IEM108 elicits more-effective antibacterial immunity than IEM101 and furthermore induces an antitoxic response.

DISCUSSION

Some live oral attenuated vaccines have been developed from strains of the O1, E1 Tor and classical biotypes, or O139 serogroup. Most candidates are derived from wild toxigenic strains and were constructed by deleting the CT gene besides other potential virulence genes, such as the hemolysin gene and the hemagglutinin/protease gene, and adding the *ctxB* gene to depress the reactogenicity and to elicit antibacterial and antitoxic immune responses (3, 9, 11, 13, 19, 20, 27). In our previous study, IEM101 (16, 17) was selected as a vaccine prototype from naturally nontoxigenic El Tor strains. Experiments with animal models and human volunteers showed that IEM101 is able to colonize the intestinal mucosa; induce strong immune responses in terms of specific IgA, IgG, IgM, and vibriocidal antibodies; and produce protective immunity. So we developed a candidate from the natural strains. However, the live attenuated vaccine strains have the potential of reacquiring the *ctxAB* gene by CTXΦ transfection from toxigenic strains in the environment or host intestine, thus reverting to toxigenicity (12, 29). For these reasons, we decided to develop a safer vaccine that elicits both antitoxic and antibacterial immunity and also is immune to CTXΦ infection.

Three forms of CTXΦ have been identified, $CTX^{class\Phi}$, $CTX^{ET\Phi}$, and $CTX^{calc\Phi}$. They were derived from O1 classical, O1 El Tor, and O139 Calcutta strains, respectively (5, 12). Up to now, only the $CTX^{ET\Phi}$ and $CTX^{calc\Phi}$ prophage can give rise to infectious phage particles and therefore transmit the *ctxAB* gene to nontoxigenic strains (5, 6). Molecular epidemiology surveys showed that the prophages contained in many toxigenic O139 strains are still $CTX^{ET\Phi}$ except for those in the Calcutta strains, which were $CTX^{calc\Phi}$ lysogens (2, 5, 24). Furthermore, the comparison of the *rstR*-intergenic region 2 sequences in El Tor clinical isolates from different continents over the past 25 years and the O139 strain showed that they were identical (12, 15).

Given that El Tor is the predominant biotype of the current seventh pandemic (27) and that $CTX^{ET\Phi}$ is the predominant infectious CTXΦ (the other infectious CTXΦ, $CTX^{calc\Phi}$, is

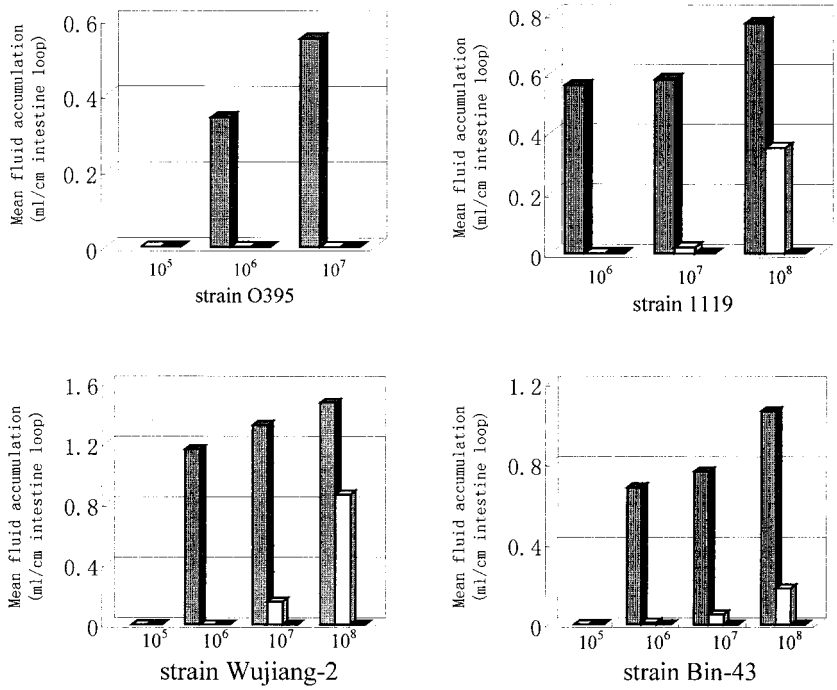

FIG. 6. Average fluid accumulations in the rabbit ileal loops after challenge with the strains O395, 1119, Wujiang-2, and Bin-43. Different animal groups are indicated as follows: naive, gray bars; IEM101, white bars; IEM108, black bars.

restricted to a few areas [5]), the *rstR* of an El Tor strain Bin-43 was introduced into IEM101 to protect the vaccine from $CTX^{ET\Phi}$ infection. The safety of IEM108 was evaluated indirectly by using a simplified model, the mating experiment, rather than a live test of CTXΦ infection. The effect of *rstR* inhibition of CTXΦ infection can be evaluated by using the transfer efficiency of the recombinant suicide plasmid containing the RS2 region of $CTX^{ET\Phi}$. The indication of repression is active expression of the El Tor-derived *rstR* gene in IEM108. If it is actively expressed, RstR should biotype specifically repress the expression of *rstA* in El Tor-derived RS2 cloned in pKRSe, thus apparently reducing the frequency of conjugation of pKRSe from SM10 to IEM108. In addition, it should not repress sufficiently the *rstA* of classical biotype-derived RS2 in pKRSn. Since there is no *rstR* in IEM101, the rates of conjugation of pKRSe and pKRSn to it were similar, and both were higher than that of pKRSe to IEM108.

Our results demonstrated that the El Tor-derived *rstR* gene cloned into IEM108 was expressed actively and could produce immunity against $CTX^{ET\Phi}$ infection. Because *rstR* is biotype specific, IEM108 acquired much stronger immunity to $CTX^{ET\Phi}$ than to $CTX^{class\Phi}$. We also found a poor cross-immunity between the El Tor-derived *rstR* and the classical biotype-derived *rstA* due to a ninefold reduction of transfer frequency of pKRSn into IEM108 compared to that into IEM101. The acquired immunity against CTXΦ in IEM108 could reduce the possibility that the vaccine strain would regain toxicity and thus enhance its safety.

It has been clear from animal studies that the presence of serum antibodies against the toxins offers protection against toxin-induced disease (1). Thus, besides the *rstR* gene, *ctxB* was also introduced simultaneously to elicit antitoxin immunological responses. Both of them were cloned into the balance plasmid containing *E. coli*-derived *thyA* to construct the improved vaccine candidate, IEM108. Though the *E. coli*-derived *thyA* gene can complement functionally the *thyA* chromosomal deletion of *V. cholerae*, the genes have no homology, which greatly diminishes the possibility of *thyA* gene recombination events, thus making the lethal balanced system more stable. It has been reported that the induced immune responses to antigens expressed from multicopy plasmids are more prominent than those to antigens expressed from single-copy genes on the chromosome (21). In our study, the *thyA* balanced system insured a higher-level expression of CTB and RstR than a single-copy gene on the chromosome. The magnitude of immune responses induced against antigens expressed by attenuated vaccine strains of *V. cholerae* is directly related to the quantity of antigen produced. So we believe that IEM108 will induce a high immune response against CT and CTXΦ infection.

Animal tests showed that IEM108 induced high anti-CTB and vibriocidal antibody responses and conferred full protection against challenge with at least 4 μg of CT and four strains of wild-type *V. cholerae*. It was found that immunity to cholera involves an antibacterial response rather than an antitoxin response, but the latter can heighten the former synergistically (14). In our study the levels of vibriocidal antibodies elicited by IEM108 were also higher than those elicited by IEM101. Moreover, the peak titer had a long duration and a slow decrease. This suggests that there is a synergistic effect between the antibacterial and antitoxin immunity produced by IEM108. Vibriocidal antibodies are a measure of immune responses against the *V. cholerae* organisms and reflect the ability of *V. cholerae* strains to colonize the intestine (23). Though the main protective antibacterial immunity is local in the intestine, high levels of vibriocidal antibodies are correlated with protection from cholera. It is normally found that the higher the vibriocidal antibody response induced, the stronger the mucosal protective immunity elicited (13). The higher vibriocidal antibody titers induced by IEM108 indicate that it is a more effective candidate than its parent strain, IEM101.

Based on our results, we can conclude that IEM108 is both immunogenic and protective. It has many properties that recommend it as a promising safe live attenuated oral vaccine candidate. These properties include the introduction of *rstR*, which efficiently renders the strain immune to $CTX^{ET\Phi}$ infection, thus providing a higher level of safety against possible reversion to enterotoxicity by reacquisition of the *ctxAB* gene; the capacity to colonize the small intestine; the high expression of the protective antigen (CTB) from a multicopy plasmid, which is stably maintained in the balanced system; and the facility with which it can be further modified, such as by introducing the *rstR* of the O139 Calcutta strain to protect against $CTX^{calc\Phi}$ infection and by introducing major protective antigen genes of O139 or other enteropathogen protective antigen genes to construct a bivalent or multivalent vaccine. Thus, it may be of interest to further evaluate IEM108 in volunteer and field testing for its efficiency and safety and the property of CTXΦ phage immunity.

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REFERENCES

1. **Acheson, D. W. K., M. M. Levine, J. B. Kaper, and G. T. Keusch.** 1996. Protective immunity to Shiga-like toxin I following oral immunization with Shiga-like toxin I B-subunit-producing *Vibrio cholerae* CVD103-HgR. 1996. Infect. Immun. **64:**355–357.
2. **Basu, A., A. K. Mukhopadhyay, C. Sharma, J. Jyot, N. Gupta, A. Ghosh, S. K. Bhattacharya, Y. Takeda, A. S. Faruque, M. J. Albert, and G. B. Nair.** 1998. Heterogeneity in the organization of the CTX genetic element in strains of *Vibrio cholerae* O139 Bengal isolated from Calcutta, India and Dhaka, Bangladesh and its possible link to the dissimilar incidence of O139 cholera in two locales. Microb. Pathog. **24:**175–183.
3. **Benítez, J. A., L. García, A. Silva, H. García, R. Fando, B. Cedré, A. Pérez, J. Campos, B. L. Rodríguez, J. L. Pérez, T. Valmaseda, O. Pérez, A. Pérez, M. Ramírez, T. Ledón, M. Díaz Jidy, M. Lastre, L. Bravo, and G. Sierra.** 1999. Preliminary assessment of the safety and immunogenicity of a new CTXΦ-negative, hemagglutinin/protease-defective El Tor strain as a cholera vaccine candidate. Infect. Immun. **67:**539–545.
4. **Butterton, J. R., S. A. Boyko, and S. B. Calderwood.** 1993. Use of the *Vibrio cholerae irgA* gene as a locus for insertion and expression of heterologous antigens in cholera vaccine strains. Vaccine **11:**1327–1335.
5. **Davis, B. M., H. H. Kimsey, W. Chang, and M. K. Waldor.** 1999. The *Vibrio cholerae* O139 Calcutta bacteriophage CTXΦ is infectious and encodes a novel repressor. J. Bacteriol. **181:**6779–6787.
6. **Davis, B. M., K. E. Moyer, E. F. Boyd, and M. K. Waldor.** 2000. CTX prophages in classical biotype *Vibrio cholerae*: functional phage genes but dysfunctional phage genomes. J. Bacteriol. **182:**6992–6998.
7. **Faruque, S. H., M. J. Albert, and J. J. Mekalanos.** 1998. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae.* Microbiol. Mol. Biol. Rev. **62:**1301–1314.
8. **Gennaro, M. L., P. J. Greenaway, and D. A. Broadbent.** 1982. The expression of biologically active cholera toxin in *Escherichia coli.* Nucleic Acids Res. **10:**4883–4890.
9. **Gotuzz, E., B. Butron, C. Seas, M. Penny, R. Ruiz, G. Losonsky, C. F.**

Lanata, S. S. Wasserman, E. Salazar, J. B. Kaper, S. Cryz, and M. M. Levine. 1993. Safety, immunogenicity and excretion pattern of single-dose live oral cholera vaccine CVD 103-HgR in Peruvian adults of high and low socioeconomic levels. Infect. Immun. **61:**3994–3997.

10. **Kan, B., G. M. Qi, and Y. Q. Liu.** 2002. Replicative and integrative functions in RS in lysogenic bacteriophage nct-CTXΦ genome without *ctxAB* in *Vibrio cholerae.* Chin. J. Microbiol. Immunol. (Beijing) **22:**435–438.
11. **Kenner, J. R., T. S. Coster, D. N. Taylor, A. F. Trofa, M. Barreraoro, T. Hyman, J. M. Adams, D. T. Beattie, K. P. Killeen, and J. C. Sadoff.** 1995. Peru-15, an improved live attenuated oral vaccine candidate for *Vibrio cholerae* O1. J. Infect. Dis. **172:**1126–1129.
12. **Kimsey, H. H., and M. K. Waldor.** 1998. CTXΦ immunity: application in the development of cholera vaccines. Proc. Natl. Acad. Sci. USA **95:**7035–7039.
13. **Levine, M. M., and J. B. Kaper.** 1995. Live oral vaccine: from principle to product. Bull. Inst. Pasteur **93:**243–245.
14. **Levine, M. M., and N. F. Pierce.** 1992. Immunity and vaccine development, p. 285–327. *In* D. Barua and W. B. Greenough III (ed.), Cholera. Plenum Medical Book Company, New York, N.Y.
15. **Liang, W. L., B. Kan, G. M. Qi, Y. Q. Liu, and S. Y. Gao.** 2003. Study of induction of the lysogenic bacteriophage CTXΦ in *V. cholerae* O139 strain and its transduction. Chin. J. Microbiol. Immunol. (Beijing) **23:**53–56.
16. **Liu, Y. Q., G. M. Qi, S. X. Wang, Y. M. Yu, G. C. Duan, L. J. Zhang, and S. Y. Gao.** 1995. A natural vaccine candidate strain against cholera. Biomed. Environ. Sci. **8:**350–358.
17. **Maria, R. F., M. Elisabetta, Y. Q. Liu, G. M. Qi, G. C. Duan, R. Rappuoli, and M. Pizza.** 2001. IEM101, a naturally attenuated *Vibrio cholerae* strain, as carrier for genetically detoxified derivatives of cholera toxin. Vaccine **19:**75–85.
18. **Parsot, C., and J. J. Mekalanos.** 1990. Expression of ToxR, the transcriptional activator of the virulence factors in *Vibrio cholerae*, is modulated by the heat shock response. Proc. Natl. Acad. Sci. USA **87:**9898–9902.
19. **Rijpkema, S. G. T., E. M. Bik, W. H. Jansen, J. H. Gielen, L. F. Versluis, A. H. Stouthamer, P. A. M. Guinee, and F. R. Mooi.** 1992. Construction and analysis of a *Vibrio cholerae* δ-aminolevulinic acid auxotroph which confers protective immunity in a rabbit model. Infect. Immun. **60:**2188–2193.
20. **Robert, A., A. Silva, J. A. Benitez, B. L. Rodriguez, R. Fando, J. Campos, D. K. Sengupta, M. Boesman-Finkelstein, and R. A. Finkelstein.** 1996. Tagging a *Vibrio cholerae* El Tor candidate vaccine strain by disruption of its hemagglutinin/protease gene using a novel reporter enzyme: *Clostridium thermocellum* endoglucanase A. Vaccine **14:**1517–1522.
21. **Ryan, E. T., J. R. Butterton, T. Zhang, M. A. Baker, S. L. J. Stanley, and S. B. Calderwood.** 1997. Oral immunization with attenuated vaccine strains of *Vibrio cholerae* expressing a dodecapeptide repeat of the serine-rich *Entamoeba histolytica* protein fused to the cholera toxin B subunit induces systemic and mucosal antiamebic and anti-*V. cholerae* antibody responses in mice. Infect. Immun. **65:**3118–3125.
22. **Ryan, E. T., and S. B. Calderwood.** 2000. Cholera vaccines. Clin. Infect. Dis. **31:**561–565.
23. **Ryan, E. T., T. I. Crean, S. K. Kochi, M. John, A. A. Luciano, K. P. Killeen, K. E Klose, and S. B. Calderwood.** 2000. Development of a Δ*glnA* balanced lethal plasmid system for expression of heterologous antigens by attenuated vaccine vector strains of *Vibrio cholerae.* Infect. Immun. **68:**221–226.
24. **Sharma, C., A. K. Maiti, A. K. Mukhopadhyay, A. Basu, I. Basu, G. B. Nair, R. Mukhopadhyaya, B. Das, S. Kar, R. K. Ghosh, and A. Ghosh.** 1997. Unique organization of the CTX genetic element in *Vibrio cholerae* O139 strains which reemerged in Calcutta, India, in September 1996. J. Clin. Microbiol. **35:**3348–3350.
25. **Simon, R. U., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology. **1:**784–791.
26. **Svennerholm, A. M., D. A. Sack, J. Holmgren, and P. K. Bardhan.** 1982. Intestinal antibody responses after immunization with cholera B subunit. Lancet **i:**305–308.
27. **Taylor, D. N., K. P. Killeen, D. C. Hack, J. R. Kenner, T. S. Coster, D. T. Beattic, J. Ezzell, T. Hyman, A. Trofa, M. H. Sjogren, A. Friedlander, J. J. Mekalanos, and J. C. Sadoff.** 1994. Development of a live, oral, attenuated vaccine against El Tor cholera. J. Infect. Dis. **170:**1518–1523.
28. **Waldor, M. K., E. J. Rubin, G. D. N. Pearson, H. Kimsey, and J. J. Mekalanos.** 1997. Regulation, replication, and integration functions of the *Vibrio cholerae* CTXΦ are encoded by region RS2. Mol. Microbiol. **24:**917–926.
29. **Waldor, M. K., and J. J. Mekalanos.** 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science **272:**1910–1914.
30. **Xia, X. B., G. M. Qi, and Y. Q. Liu.** 2000. Development of a chromosome-plasmid balanced lethal gene expression of *Vibrio cholerae* based on *thyA* locus. Chin. J. Microbiol. Immunol. (Beijing) **20:**223–227.

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