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Resistance of the cholera vaccine candidate IEM108 against CTXΦ infection

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Abstract

The cholera toxin (CT) genes *ctxAB* are carried on a lysogenic phage of *Vibrio cholerae*, CTXΦ, which can transfer *ctxAB* between toxigenic and nontoxigenic strains of bacteria. This transfer may pose a problem when live oral cholera vaccine is given to people in epidemic areas, because the toxin genes can be reacquired by the vaccine strains. To address this problem, we have constructed a live vaccine candidate, IEM108, which carries an El Tor-derived *rstR* gene. This gene encodes a repressor and can render bacterial resistance to CTXΦ infection. In this study, we evaluated the resistance of IEM108 against CTXΦ infection by using a CTXΦ marked for chloramphenicol (CAF) resistance and an in vivo model. We found that the cloned *rstR* gene rendered IEM108 immune to infection with the marked CTXΦ. In addition, the infection rate of IEM108 was even lower than that of the native CTXΦ-positive strain. These results suggest that the vaccine candidate IEM108 is resistant to infection by CTXΦ.

Keywords: *Vibrio cholerae*; Vaccine; Phage infection

1. Introduction

Toxigenic strains of *Vibrio cholerae* have triggered seven cholera pandemics in the world since 1817 [1] and remain a menace to public health in developing countries. Cholera toxin (CT), encoded by the *ctxAB* genes, is the principal virulence factor of *V. cholerae*. Toxigenic *V. cholerae* strains are lysogens of a filamentous bacteriophage designated CTXΦ, which carry the *ctxAB* genes [2]. The CTXΦ genome is divided into a 4.6-kb core region containing the genes required for virion morphogenesis as well as *ctxAB* and a 2.4-kb RS2 region containing the genes *rstA*, *rstB* and *rstR*. These latter three genes encode the regulation, replication, and integration functions of the CTXΦ genome [3]. CTXΦ can integrate its genome into the *V. cholerae* chromosome and form a stable prophage or it can replicate as a plasmid [2,3]. The integration of CTXΦ into the *V. cholerae* chromosome is site-specific and requires the chromosome-encoded XerCD [4].

Vaccination against cholera is a needed preventive measure for people threatened by cholera epidemics as well as tourists entering the areas where cholera is present. Oral vaccines are currently the dominant strategy in the development of a vaccine for cholera. The two approaches employed in developing vaccines are the use of killed whole *V. cholerae* cells plus CTB and the use of attenuated live *V. cholerae* strains [5,6].

CTXΦ may transfer *ctxAB* from a toxigenic strain to a nontoxigenic strain through lysogenic conversion [2]. Thus, the potential exists for the vaccine strains to regain *ctxAB* by horizontal transfer of CTXΦ when a live oral vaccine is used in cholera epidemic areas. In these regions, large numbers of

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live bacterial strains are released into the environment. This release increases the probability of contact between the vaccine strains and the environmental toxigenic strains. Therefore, the safety of the live vaccine is an important aspect to be considered to prevent further spread of CTXΦ infections.

RstR, the protein encoded by the *rstR* gene in the RS2 region, is a repressor of CTXΦ. This protein mediates the super-immunity of CTXΦ super-infection. This feature of RstR can be used to design a cholera vaccine safe from infection by CTXΦ [7]. RstR represses the transcription of *rstA* and *rstB*, and thus blocks the replication and integration of a secondary infection of CTXΦ [7,8]. Different varieties of the CTXΦ phage exist in different biotype or serotype strains. They are distinguished based on their different *rstR* and *rstA* operator sequences and on the presence or absence of *ctxAB* [7,9–12]. There is no cross repression of *rstR* among different CTXΦs having different *rstR* sequences because of the dissimilar RstR-binding sequences in the *rstA* promoter region [9].

V. cholerae El Tor is currently the main epidemic biotype. Therefore, it is important to focus our studies on this biotype. We have developed a live vaccine candidate, IEM108, which provided immunity to CTXΦ infection [13]. IEM108 was constructed by introducing the *ctxB* gene and the El Tor-derived *rstR* gene into its prototype strain IEM101 [14,15]. The safety of IEM108 against El Tor-derived CTXΦ (denoted $CTX^{ET}\Phi$) infection was estimated using a conjugation model [13]. The recombinant plasmids used in that model contained the suicide vector and the native regulatory region of the CTXΦ genome, RS. Because the RS region is required for replication and integration of CTXΦ, infection by CTXΦ may be indicated indirectly through the integration of the recombinant plasmids in conjugating *V. cholerae* strains. In this study, we established an in vivo infection model using the entire CTXΦ genome marked with the chloramphenicol (CAF) resistance gene (*cat*). This approach allowed us to directly evaluate the repression efficacy of *rstR* encoded in IEM108. Our results show that IEM108 had the lowest infection rate of the marked CTXΦ among the test strains, even lower than the wild type CTXΦ lysogenic El Tor strain. This suggests that IEM108 could be a live vaccine candidate with sufficient safety to prevent CTXΦ infection.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The vaccine candidate IEM108 was previously described [13], as was its prototype El Tor biotype strain IEM101 (CTXΦ-negative, Ogawa) [14]. Native CTXΦ-positive strains 1119 (classical biotype, Inaba) and Wujiang-2 (El Tor biotype, Inaba) from patients are utilized in our laboratory. N16961 (El Tor, Inaba) and the *Escherichia coli* strain S17-1λ*pir* (used to maintain the suicide plasmid pCVD442) were a gift of Dr. J.B. Kaper of the University of Maryland. Strain N-Φc (derived from N16961, Δ*ctxAB*::*cat*, CAF^r) was constructed for this study.

All strains were cultured in Luria-Bertani (LB) broth unless otherwise noted. Antibiotics and concentrations were as follows: ampicillin (AP), 100 μg/ml; chloramphenicol (CAF), 15 μg/ml (for strains of *V. cholerae*); CAF, 50 μg/ml (for strains of *E. coli*); streptomycin, 10 μg/ml; and gentamycin, 0.5 U/ml. The sucrose-resistant strains were selected on a modified LB agar medium (without NaCl) containing 10% sucrose as described [16].

2.2. Molecular methods

PCR was performed and DNA fragments were cloned using standard protocols [17]. Colony hybridization and Southern blot were used to detect and verify the presence of the genes on chromosomal and plasmid DNA. In colony hybridization, nitrocellulose membranes with bacterial colonies were removed from the agar plates, the bacteria were lysed, and their DNA was denatured and fixed to the membranes as described [17]. For Southern blot analysis, the DNA fragments were transferred onto the nitrocellulose membranes after separation by gel electrophoresis. Hybridizations using a random-primer DIG DNA labeling and detection kit (Roche Molecular Biochemicals, Germany) were performed according to the manufacturer's directions. Conjugation transfer of the recombinant suicide plasmid from *E. coli* strain S17-1λ*pir* to the recipient *V. cholerae* was performed as previously described [13].

2.3. Construction of plasmids and strains

Plasmids used in this study are described in Table 1. The plasmid pCTR5A, previously constructed in our laboratory, contains the *ctxAB* gene and its upstream (*zot*) and downstream (RS) fragments of $CTX^{ET}\Phi$, were used as the foundation to generate a homologous fragment containing *cat* instead of *ctxAB*. pCTR5A was digested with *Hinc*II and *Xba*I, resulting in two fragments of 4.4 kb and 920 bp. The 920 bp fragment within the *ctxAB* open reading frame, was removed and replaced with the 800 bp *cat* gene, thus generating p5Acat. The *cat* fragment was amplified using pCOS5

Table 1
Plasmids used in this study

Plasmids	Description	Source
pCTR5A	pCT5A11 [18] derived, containing *ctxAB* and partial sequences of its upstream (*zot*) and downstream (RS) of $CTX^{ET}\Phi$, AP^r	Our lab
pCOS5	Cosmid vector, *oriT*, AP^r, CAF^r.	[19]
p5Acat	pCTR5A derived, *ctxAB* fragment was replaced by *cat*, AP^r, CAF^r	This study
pCVD442	Suicide plasmid vector, $sucrose^S$ *mob*, *ori*, *bla*, *sacB*, AP^r	[16]
p4Φc	pCVD442 derived, containing *cat* with its upstream (*zot*) and downstream (RS) sequences in p5Acat, AP^r, CAF^r	This study

[19] with primers Pcat1 (CGT AGC ACC AGG CGT TTA AG) and Pcat2 (GAT CGG CAC GTA AGA GGT TC). The p5Acat was digested with *Pst*I and *Pvu*I, then blunt-ended with T4 DNA polymerase and further digested with *Sac*I. The result was a 2.5 kb fragment that included *cat* and its flanking sequences (*zot* and RS). This 2.5 kb fragment was purified by agarose gel electrophoresis and ligated into the *Sma*I and *Sac*I-digested suicide vector pCVD442 to generate the recombinant suicide plasmid, named p4Φc. This plasmid contains the *cat* gene and the upstream and downstream regions homologous to the flanking sequences of *ctxAB* of the $CTX^{ET}\Phi$ genome.

The *E. coli* strain JM109 was used for general transformations during cloning, and S17-1λ*pir* was used as the recipient strain of the recombinant suicide plasmid p4Φc. S-17λ*pir*, carrying p4Φc, was used as the donor strain when it was mated with N16961 by conjugation. After homologous recombination mediated by p4Φc, the *cat* gene on p4Φc replaced *ctxAB* of the CTXΦ genome in the N16961 chromosomal DNA, resulting in the CTXΦ mutant genome N-Φc. This *cat*-marked CTXΦ phage, designated CTXΦc, could be isolated from mitomycin-C induced cultures of the strain N-Φc. After their isolation, we used the induced CTXΦc phage particles to infect IEM108, 1119, Wujiang-2, and IEM101 to calculate and compare the infection rates among these strains.

2.4. In vitro and in vivo CTXΦc infection assays

To obtain CTXΦc particles, the strain N-Φc was cultured in LB containing 20 ng/ml mitomycin-C at 30 °C for 6–7 h. The supernatant of the culture was collected by centrifugation (15 min, 6300 × *g*) at 4 °C, followed by filtration through a 0.22 μm membrane. The resulting filtrate was checked for the presence of contaminating cells by plating 0.2 ml on LB agar. The phage particles were separated from the fluid by adding PEG8000 and NaCl into the supernatant to a final concentration of 20% (PEG8000) and 10% (NaCl). The supernatant mixture was then centrifuged at 6300 × *g* for 30 min at 4 °C. The pellet was dissolved in TES as described [20] and stored at 4 °C.

For the CTXΦc in vitro infection assay, *V. cholerae* strains were inoculated without shaking in colonization factor broth at 30 °C for 18–24 h, as described previously [20]. These conditions induced the expression of the CTXΦ receptor, TCP [2]. The bacterial strains IEM108, 1119, Wujiang-2 and IEM101 were then individually mixed with CTXΦc, and cultured without shaking at 30 °C for 3 h. The resulting infected clones were selected on LB plates containing CAF to assess the amount of infection of each strain by CTXΦc. Simultaneously, serial dilutions of the mixtures were cultured on antibiotic-free LB plates to count the total of the infected and non-infected strains in the primary mixture. These values allowed us to calculate the frequency of the infection rate of CTXΦc in the tested strains.

Adult New Zealand White rabbits (2–2.5 kg) were used for in vivo CTXΦc infection assays. After fasting for 24 h, the rabbits were anesthetized with ether. The small intestines were then separated under sterile conditions and tied into 4–5 cm long loops. The different mixtures of the *V. cholerae* strains IEM108, 1119, Wujiang-2 and IEM101 with induced CTXΦc particles were injected into different ileal loops. The rabbits were sacrificed 10 h later. The small intestine was dissected out and the mucous membrane of each loop was washed with 1 ml of 0.9% saline. For loops that had accumulated fluid, we extracted 1 ml of liquid directly as the ileal loop fluid. The infected strains in each loop fluid sample were selected on LB agar plates containing CAF and gentamicin and the strain identity was confirmed with the corresponding standard antisera of *V. cholerae*. Some randomly selected colonies were also detected with *cat* amplification, to further verify the infection. In addition, the infection rates were calculated as described above with the sum of the infected and non-infected *V. cholerae* cells obtained by plating serial dilutions of the loop fluid onto the gentamicin-containing agar [13].

3. Results

3.1. The CTXΦ genome in N16961 was marked with cat gene

N-Φc was constructed by replacing the *ctxAB* gene with the *cat* gene in the CTXΦ genome of strain N16961. To accomplish this, we first conjugated N16961 with S17-1λ*pir* (p4Φc). The recombinant suicide plasmid p4Φc was introduced into N16961 and could integrate into the N16961 chromosomes through homologous recombination. After the homologous exchange, cells were selected based on their sucrose and CAF resistance to obtain only cells in which the suicide plasmid DNA had been excised and the *ctxAB* genes had been replaced by the *cat* gene. We obtained putative conjugates and screened these further to identify strain N-Φc.

For this identification, we tested the colonies for agglutination with the standard diagnostic sera of anti-*V. cholerae* serogroup O1 and Inaba as N16961. Colonies displaying agglutination were further verified when we amplified the *cat* gene with specific primers. In brief, *ctxAB* amplification was negative for strain N-Φc and the extracted total cellular DNA hybridized with a *cat* gene probe, but not with *ctxAB* or pCVD442 DNA probes (Fig. 1, left). Identification of strain N-Φc was further achieved by testing if the fragment amplified from strain N-Φc with primer pairs located in the flanking *zot* and RS sequences was digested into two fragments with *Eco*RI. *Eco*RI has a unique site in the *cat* sequence. This site is absent in the *ctxAB* sequences. Finally, the identity of strain N-Φc was further confirmed when its chromosomal DNA was digested with *Mlu*I and hybridized with the *cat* probe. We found that the hybridizing fragment shifted to 11 kb as expected from the predicted corresponding region of the N16961 genome after conjugation (Fig. 1, right).

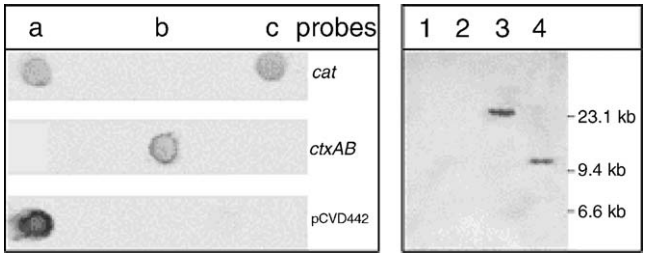

Fig. 1. Identification of the mutant strain N-Φc. Left: colony hybridization with probes of fragments of *cat*, the deleted fragment of *ctxAB* and plasmid DNA of pCVD442. The strains were 17-4Φc (a, S17-1λ*pir* carrying p4Φc), N16961 (b), and N-Φc (c). Right: Southern blot of the extracted plasmid and total genomic DNA from N-Φc, hybridized with the *cat* gene probe. The blot shows the hybridization result of extracted plasmid with (lane 2) or without (lane 1) digestion by *Mlu*I, and the total genomic DNA with (lane 4) or without (lane 3) digestion by *Mlu*I.

3.2. IEM108 resisted the infection of CTXΦc more efficiently compared with IEM101

We infected the vaccine candidate IEM108 and the other control strains with the induced CTXΦc to evaluate their resistance to infection with CTXΦc. For each infection experiment with these strains, the same CTXΦc extract was used. To infect strains 1119, Wujiang-2, IEM101 and IEM108 in vitro, we plated the mixtures of the induced CTXΦc particles and each strain on LB agar containing CAF. We detected the infected strains through dot blot hybridization with the *cat* gene probe. We found that only the classical strain 1119 could be infected by CTXΦc, albeit with extremely low frequency. The average infection rate for this strain was 1.57×10^{-7} (95% confidence interval, 8.17×10^{-8} to 3.02×10^{-7}).

The in vitro infection was followed by in vivo CTXΦc phage infection. The small intestine provides an appropriate environment for the growth of the *V. cholerae* strain and for the infection of CTXΦ [2]. We used rabbit ileal loops as the in vivo infection model of CTXΦc. For this in vivo infection, samples of the ileal fluid were plated on selective agar. Some of the resulting colonies were randomly selected and verified with the antisera, with *cat*- and *ctxAB*-specific PCR, and by dot blot hybridization. We found all of the detected colonies to be positive in these tests except for the amplification of *ctxAB*, which was negative. Subsequently, the colonies growing on the selective media were counted as infected.

Our data showed that the classical *V. choleae* strain 1119, was much more susceptible to infection with the El Tor-derived CTXΦc in vivo than were the other El Tor strains (Fig. 2). Under in vivo conditions, the geometric mean of the infection frequency for vaccine candidate IEM108 was about 1000 times lower than that of its prototype IEM101 (Fig. 2). These results suggest that the vaccine candidate IEM108 has effective immunity against phage CTXΦ infection.

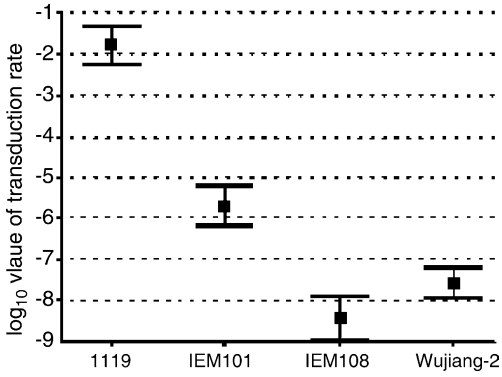

Fig. 2. In vivo infection rates of *V. cholerae* strains with CTXΦc. The infection experiments using the four strains were repeated 13 times. The statistics software used for data processing was SPSS10.0. The means of the rates and their 95% confidence intervals were: 1119, 1.61×10^{-2} (5.61×10^{-3}, 4.63×10^{-2}); IEM101, 2.02×10^{-6} (6.73×10^{-7}, 6.07×10^{-6}); IEM108, 3.05×10^{-9} (1.06×10^{-9}, 1.03×10^{-8}); and Wujiang-2, 2.73×10^{-8} (1.10×10^{-8}, 6.60×10^{-8}).

3.3. The CTXΦc genome existed as the chromosomal integration and in plasmid form in the different infected strains

*Mlu*I has a single recognized site in the CTXΦc genome. This site is located within *zot* gene. To determine the form of the CTXΦc genome present in infected strains, we digested the total DNA extracted from the infected strains with *Mlu*I and conducted a Southern blot assay with a *cat* probe. If the CTXΦc genome existed as the plasmid form in these cells, it would generate a 7.5 kb band when hybridized with the *cat* gene probe. Integration into the bacterial chromosome would produce a larger hybridization fragment. For strains 1119 and Wujiang-2, the hybridization patterns of total DNA and plasmid extracts were the same and their digested results showed only one 7.5 kb band. Therefore, in these two strains, the CTXΦc genome existed as the single plasmid. In contrast, for IEM101 and IEM108, there were 7.5 kb bands in all of the hybridization patterns of the extracts of both total DNA and plasmid DNA, when it was digested with *Mlu*I. But also, the larger hybridization fragments (about 11 kb) were also found in the total DNA extracts of both these strains (Fig. 3). The smaller fragments (about 5 kb) were found in the hybridization patterns of total DNA and plasmid extracts of these two strains without digestion with *Mlu*I but disappeared after digestion (Fig. 3). These 5 kb bands should be the supercoiled plasmid form of CTXΦc. Therefore, the CTXΦc genome was integrated into the chromosomes and also existed in the plasmid form in both the infected IEM101 and the infected IEM108 bacterial strains. There are some possible reasons for this phenomenon: CTXΦ genomes are present in the chromosomes of strains 1119 and Wujiang-2 natively. Thus, these two strains would be immune to this phage and would resist the chromosomal integration of CTXΦc. Although the CTXΦ integration site attB exists in the chromosomes of both strains, there is no CTXΦ in IEM101 and IEM108. Therefore,

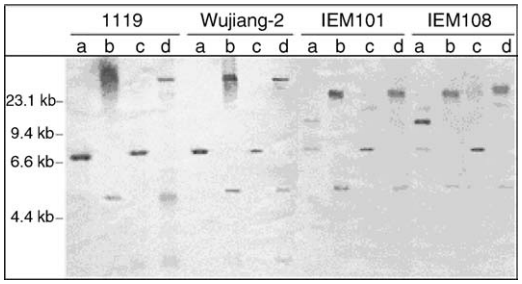

Fig. 3. Southern blot of the infected strains of different *V. cholerae* probed with the *cat* gene, to analyze the form of the CTXΦc genome present in those strains. DNA samples were extracted and treated as follows: (a) total DNA of the infected strains digested with *Mlu*I; (b) total DNA of the infected strains without digestion; (c) plasmid DNA extracted from the infected strains, digested with *Mlu*I; and (d) plasmid DNA extracted from the infected strains, without digestion. This shows that the CTXΦc genome existed only as the plasmid form in strains 1119 and Wujiang-2, while in the infected strains of IEM101 and IEM108, both the chromosomal integration and plasmid forms of CTXΦc were present.

the chromosomes of these two strains retain the potential to integrate CTXΦc.

4. Discussion

We previously developed a live oral cholera vaccine candidate, IEM108, into which the El Tor-derived *rstR* gene was introduced, making this strain resistant to infection by the $CTX^{ET}\Phi$ [13]. In our current study, we marked CTXΦ with antibiotic resistance and used the induced phage and an in vivo model to estimate the capacity of the *rstR*-containing IEM108 to inhibit infection by the marked phage CTXΦc. Previously, we used a conjugation and integration model with the recombinant suicide plasmid containing the RS region of CTXΦ to estimate the *rstR*-mediated inhibition of integration of the CTXΦ genome into IEM108 [13]. In this study, when considering either replication or integration we found that the new phages in this current model resemble the natural infection of native phages more closely than did the recombinant plasmids used in past studies. We found that infection was inhibited in the animal intestinal environment in vivo. Thus, our results suggest that our vaccine candidate IEM108 is resistant to CTXΦ infection.

CTXΦ infects *V. cholerae* by using TCP as its receptor. The expression of TCP by the cell is a prerequisite for its susceptibility to CTXΦ and the expression level is an important factor affecting the infection frequency [2]. Our data show that the infection rate of the classical strain 1119 with CTXΦc was much higher than that of the El Tor strains. We also found that the infection rates in vivo were much higher than those in vitro, corroborating results obtained in the previous study [2]. TCP is known to be expressed more efficiently within gastrointestinal tracts compared with laboratory culture conditions. In contrast to the previous report [2], we did not obtain any infected strains of the El Tor *V. cholerae* in vitro. Possible explanations for this difference may be altered culture conditions or the use of different strains. Infection rates for our in vivo model, however, were high enough to be compared among the different strains.

The repression of *rstR* is biotype-specific. For example, classical-derived *rstR* represses classical-derived CTXΦ (denoted $CTX^{class}\Phi$) and El Tor-derived *rstR* represses $CTX^{ET}\Phi$ [7]. For the two CTXΦ lysogens 1119 and Wujiang-2, the classical-derived *rstR* exists in 1119, while the El Tor-derived *rstR* is present in Wujiang-2. Hence, CTXΦc showed a much higher infection rate in 1119 than in the other strains. The rate of infection of Wujiang-2 is very low because the repression mechanism mediated by the identical biotype-derived *rstR* exists in Wujiang-2. We compared the CTXΦc-infection rate of IEM108 with its wild prototype IEM101. IEM101 has none of the CTXΦ genome while its derivative, IEM108, includes *rstR* which was introduced into it. Although CTXΦc could transfect IEM108, its infection rate was 10^3 times lower than that of IEM101 due to *rstR*-mediated repression. The infection rate of CTXΦc to IEM108, as well as Wujiang-2 which carry El Tor-derived *rstR*, is not null. It is reported [21] that CTXΦ lysogens may also contain plasmid form of it. In another study [9] the replicative forms of $CTX^{ET}\Phi$ and $CTX^{calc}\Phi$ were transformed with the lower rates into the recipients, which were integrated with the same type of CTXΦ. We assumed that the preexistent *rstR* might permit the existence of plasmid form of its own prophage genome and the foreign CTXΦ with the identical *rstR* under some unknown status in some cells. However, in our study the ratios of such recipients were extremely low.

For Wujiang-2, the infection rate was lower than that of IEM101 because CTXΦ was already present on its chromosome. The data of infection rate of IEM108 was also 10 times lower than that of Wujiang-2. The construction of IEM108 was based on the idea of the plasmid-chromosome lethal balance system, using the housekeeping gene *thyA*. *rstR* is carried on the balance plasmid pUC19 [13]. We assumed that the number of *rstR* copies in Wujiang-2 is lower than in IEM108, since the *rstR* in Wujiang-2 is on the chromosome. In addition, the *rstC* gene in RS1 expresses an antirepressor that counteracts the activity of the phage repressor RstR, by binding directly to the RstR protein and forming insoluble and stable complexes [22]. Wujiang-2 carries the *rstC* gene, while IEM108 does not. All of these considerations may contribute to the increased degree of immunity to CTXΦc infection of *rstR*-containing IEM108 relative to Wujiang-2. The TCP expression of these two strains, other than these explanations, should be considered as well. The level of TCP expression of these two strains may also be one of the factors for the difference of infection rates.

The CTXΦ genome may exist as a chromosomal integration or in plasmid form after infection of *V. cholerae* strains by the phages [23]. Our Southern blots showed that the CTXΦc genome integrated into the chromosomes of IEM101 and IEM108 and was also present as the plasmid form, whereas it replicated only as the plasmid form in 1119 and in Wujiang-2. Previously, we also found that the RS region of $CTX^{class}\Phi$

may mediate the chromosomal integration and the plasmid form in the CTXΦ-negative strain [24]. CTXΦ integrated into the *V. cholerae* chromosome at a specific attachment site known as attB [4,25]. It is reported that [9] the second heterogenous CTXΦ may infect the CTXΦ lysogen and integrate subsequently into chromosome DNA at the integration site of 3′ ER of the first one. Although the specific site exists in both the classical strain 1119 and Wujiang-2, CTXΦc (El Tor-derived) did not integrate in either of these strains. The possible explanation is that the *rstR* genes of CTXΦc and the CTXΦ prophage in Wujiang-2 are the same and the integration of CTXΦc is inhibited strongly. Strain 1119 is a classical biotype, CTXΦc presented as plasmid and no integration into its chromosome. It has been reported that $CTX^{ET}\Phi$ presents as the plasmid replicative form in the classical strain [2], further studies are needed to understand why a newly-entering heterogenous CTXΦ genome is unable to integrate in tandem with the existing classical-derived prophage.

To construct a vaccine candidate resistant to CTXΦ infection, some other approaches may also be considered, such as to delete *recA* [26,27] to diminish the reacquisition of toxigenic elements by homologous recombination, and to delete the integration site of attB [28], to prevent the integration of CTXΦ. TCP pili, the receptor of CTXΦ, may also be the consideration to prevent reacquisition of the cholera toxin genes, whereas it is necessary for the colonization of *V. cholerae* in intestine and inducement of protective immunity. Possibly the motifs of TCP should be elaborated where is responsible for the CTXΦ attachment but unnecessary for its structure and functions of colonization and inducement of protective immunity, then such motif were modified to prevent the attachment of CTXΦ, whereas the colonization function of TCP could be preserved. Such genetic modified TCP could be used in the vaccine candidate to prevent infection of different CTXΦs.

Up to now more than 4 CTXΦ prophage types with diverse *rstR* genes have been found in different strains [29]. Except for the Calcutta CTX prophage ($CTX^{calc}\Phi$) exists in some serogroup O139 Calcutta strains [9], most El Tor and O139 strains carry $CTX^{ET}\Phi$ [7,29], therefore in this study the resistance to $CTX^{ET}\Phi$ infection of IEM108 carrying El Tor type of *rstR* was assessed. The vaccine candidates, which are resistant to the infection of other CTXΦ types, might be constructed with the similar strategy. Before a new comprehensive strategy to prevent infection of all CTXΦ types become feasible (such as the approach of TCP modification to prevent the infection of all CTXΦ types and preserve the ability of colonization were successful), it may be considered that the current epidemic type(s) of *rstR*, based on the epidemiological surveillance, can be selected to develop the vaccine. It seems to be simplified in strategy that all types of *rstR* are transferred into one vaccine strain, but the efficacy and practicability should be discussed.

In summary, we evaluated the resistance of the cholera vaccine candidate IEM108 against $CTX^{ET}\Phi$ infection in vivo. Our evaluation showed an infection rate for IEM108 of 10^{-9}, while that for the classical strain 1119 was 10^{-2}. Thus, IEM108 is a safe *V. cholerae* vaccine candidate for $CTX^{ET}\Phi$ immunity in that this strain effectively resists infection by CTXΦ. Furthermore, the model established in this study could be used for safety evaluations needed to prevent the possibility of live cholera vaccine reacquiring the gene for the toxin.

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