# Construction and Characterization of a *thyA* Mutant Derived From Cholera Vaccine Candidate IEM101

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## Abstract

A naturally cholera toxin gene negative *Vibrio cholerae* (O1, El Tor, Ogawa) strain, named IEM101, was isolated in China. The human volunteer tests showed that this strain was safe, able to colonize the intestinal mucosa, and able to induce a strong immune response. Also other studies indicated that it was an efficient live vector to deliver heterologous antigens. In this article, a thymidylate synthase gene (*thyA*)-defined mutant was constructed using homologous recombination. Except for the morphological changes in minimal medium and slightly reduced colonization capacity, mutant strain IEM101-T maintained most of the desirable features as the wild-type strain IEM101 in terms of growth rate and immunogenicity. However, the mutant was more biosafe than its parent strain. In conclusion, IEM101-T may be a promising strain to develop live vaccine candidate of cholera or an attractive vaccine vector to deliver heterologous antigens in vivo.

**Index Entries:** *Vibrio cholerae*; *thyA*; homologous recombination; mutant; vaccine.

## 1. Introduction

The acute diarrheal disease cholera remains a significant public health problem, causing more than five million cases and 200,000 deaths annually in the world ***(1)***. *Vibrio cholerae*, the causative agent of cholera, is transmitted by contaminated water and food. The explosive epidemic nature and severity of the disease and the potential threat to food and water supplies have prompted the listing of *V. cholerae* as an organism in biological defense research ***(2)***. Live oral cholera vaccines seem the most promising for eliciting of multifactorial and long-lasting immunity after a single dose. However, the implicit release of living bacteria into the environment continues to be a cause of concern worldwide.

A key enzyme involved in the biosynthetic pathway of bacteria is thymidylate synthase (TS), which is responsible for the catalytic conversion of dUMP into dTTP ***(3)***. The gene encoding for this enzyme is named *thyA* and has been studied in many bacteria ***(4–7)***. Bacterial strains bearing deletion within the *thyA* gene are auxotrophic for thymidine and are not supposed to proliferate in the environment, where free pyrimidines are absent. Inactivation of *thyA* gene has been proposed as a biological containment tool for microorganisms intended to be released into the environment.

Previous to this work, undefined mutants of *V. cholerae* with thymidine requirements had been selected by trimethoprim resistance. For example, CVD102, a spontantous *thyA*-defective derivative of CVD101, was poorly excreted by humans and minimally immunogenic ***(8)***. Most importantly, the potential reversion of mutation with unkown mechanism could lead to recovery in virulence. Nevertheless, efforts have been made to diminish the possibility of the event. For instance, Valle et al. ***(9)*** have recently demonstrated that the internal

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Table 1
Bacterial Strains and Plasmids Used in This Study

| Strain or Plasmid | Description | Reference |
|---|---|---|
| *E. coli* | | |
| DH5α | K12 cloning host strain | Lab stock |
| SM10λpir | *recA::RP4-2Tc::MuλpirR6K), Km*$^r$ | ***(12)*** |
| *V. cholerae* | | |
| IEM101 | El Tor ,Ogawa, Δ*(ctx ace zot),*wild-type, Get$^r$ | ***(10)*** |
| IEM101-T | *thyA* mutant of IEM101 | This study |
| Plasmids | | |
| pUC18 | General cloning vector, laz$^+$, Amp$^r$ | Lab stock |
| pKTN701 | mob RP4 ori R6K Cm$^r$ | ***(12)*** |
| pFGY101 | pUC18::*thyA* | This study |
| pFGY102 | pUC18:: Δ*thyA* | This study |
| pFGY103 | pKTN701:: Δ*thyA* | This study |

deletion of the *thyA* gene of O1 *V. cholerae* vaccine candidateds to enhance the environmental safety features without a detrimental effect on their colonizing and immunizing properties in animal models.

In this article, we describe the construction and characterization of a *thyA*-defined mutant derived from our own vaccine candidate of strain IEM101 ***(10)***.

## 2. Materials and Methods

### 2.1. Bacterial Strains, Plasmids, and Media

The bacterial strains and plasmids used in this study were listed in **Table 1**. *Escherichia coli* strain DH5α was used for cloning purpose. *E. coli* SM10λpir strain was used as donor strain for conjugation. *V. cholerae* strain IEM101 was used as recipient of the plasmid-containing mutant *thyA* gene for chromosomal integration. All bacterial strains were routinely grown in LB medium or agar plates with antibiotics when required. The antibiotics and supplements were added at the following concentrations: ampicillin, 100 μg/mL; gentamicin, 0.75 μg/mL; chloramphenicol, 15 or 25 μg/mL, thymidine 50 μg/mL or 200 μg/mL when necessary.

### 2.2. Genetic Methods

Isolation of genomic or plasmid DNA, transformation, agarose gel electrophoresis, and Southern blot were performed by standard molecular biological techniques ***(11)***. DNA restriction and modification enzymes were used according to the manufacturer's instructions (Sino America Biology, China). Dig-labeled probes were generated with the DNA labeling and detection kit (Roche, Germany).

### 2.3. Cloning of thyA Gene From V. cholerae IEM101

A single clony of *V. cholerae* IEM101 was picked up from plate and resuspended in 20 μL of distilled water and boiled for 10 min. One microliter of supernatant was used as template for 100 μL of *thyA* PCR reactions. On the basis of published *thyA* sequence of *V. cholerae* strain VCH6514 (GenBank accession no. AJ006514), the oligonucleotides P1 (5'-GCCATCCTTCACAGCTTT-3') and P2 (5'-CG*GAATTC*ACGGGATTAGA CTG AAAAC-3') were used as forward and reverse primers, respectively. P1 corresponds to the 5' untranslated region and a intrinsic *Eco*RI site is inculed, whereas P2 contains the stop codon and a introduced *Eco*RI site (italic sequence, a graphic representation of oligonucleotides is given in **Fig. 1**). The PCR reaction was subjected to 30 temperature cycles (94° for 1 min; 52° for 1 min; 72° for 1 min). The amplified 1165-bp fragment was digested with *Eco*RI and cloned into pUC18 digested with *Eco*RI, yielding pFGY101.

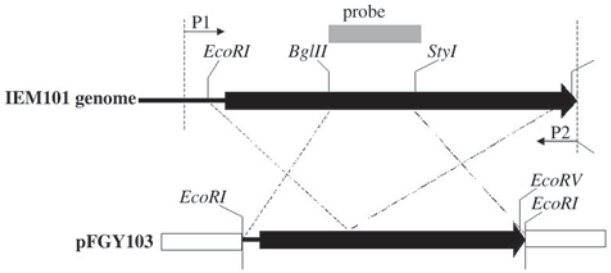


Fig. 1. Schematic diagram for *thyA* locus of *V. cholerae* IEM10 and construction of pFGY103.Open reading frame of *thyA* gene are depicted by arrows, which indicate its orientation, and relevant restriction sites are also indicated. P1 and P2 show the positions of primers used for PCR amplification of *thyA* gene and its 5' flanking region, and a *Eco*RI site was introduced by P2, which is underlined. For homologous recombination purpose, plasmid pFGY103 was constructed by the insertion of *thyA* PCR fragment with an internal 540-bp *Bgl*II–*Sty*I deletion into suicide vector pKTN701, which is showed by open box in panel B. The *thyA*-specific probe was recovered from *Bgl*II–*Sty*I digestion of plasmid pFGY101, and it is indicated by a gray box in the top of the figure. Dashed lines represent the recombination events.

### 2.4. Sequencing of thyA Gene

Plasmid DNA of pFGY101 was sequenced and the nucleotide sequence, protein alignment were analyzed with software DNAStar.

### 2.5. Construction of thyA-Defined Mutant of V. cholerae IEM101

The IEM101 *thyA* mutant was generated by allelic replacement through homologous recombination (***12***, **Fig. 1**). Briefly, following the deletion of the 535-bp *Bgl*II–*Sty*I fragment from the *thyA* gene coding region in plasmid pFGY101, a larger fragment containing mutated *thyA* gene was recovered by gel extraction, blunted, and self-ligated to generate pFGY102. An *Eco*RI fragment from pFGY102 containing this mutated *thyA* gene was cloned into suicide vector pKTN701 to obtain pFGY103. pFGY103 were mobilized from donor strain SM10λpir to receptor *V. cholerae* strains by conjugation at a donor-to-receptor strain ratio of 1:9. *V. cholerae* conjugates were selected for the first recombination event on LB agar plate with 15 μg/mL Chloramphenicol and 0.75 μg/mL of gentamycin. To select for the second recombinant event, in which the suicide vector pKTN701 was deleted from the chromosome, the chloramphenicol-sensitive enrichment procedure was used as previously described with a little modification by increasing the concentration of chloramphenicol to 25 μg/mL and D-cycloserine (Sigma) to 2000 μg/mL ***(13)***. Cointegrates from chloramphenicol-sensitive enrichment were serially diluted and subcultured on LB agar with 50 μg/mL of thymidine and 0.75 μg/mL of gentamycin. Colonies of gentamycin-resistant, chloramphenicol-sensitive and thymidine-dependence were collected and further verifed by PCR and Southern blotting for the structure of *thyA* locus. Briefly, using P1 and P2 as primer set and genomic DNA from mutant and wild-type as templates, PCR was performed as previously described. Genomic DNA from mutant and wild-type was digested with *Eco*RI or *Pst*I and Southern blotting was carried out as standard method ***(11)***. The excised 535-bp *Bgl*II–*Sty*I fragment from the *thyA* gene coding sequence was dig-labeled and used as prob. The *V. cholerae* colony in which the mutated allele replaced the wild-type *thyA* exhibited dependence on thymidine for growth in LB medium. One such mutant was designated IEM101-T and further characterized in vitro and in vivo.

### 2.6. Phenotypical Examination of IEM101-T

Agglutination serotyping was performed on a slide with monoclonal antibodies against O antigen of *V. cholerae* by standard procedure ***(14)***. The thymidine requirement of IEM101-T was examined by culturing in LB supplemented with various concentrations of thymidine. A growth curve of wild-type IEM101 and mutant IEM101-T was plotted by measurement of optical density every 30 min until the stationary phase of growth, and the medium used here were LB and LB with 50 μg/mL thymidine, respectively. Appropriate samples were taken at each interval and Gram-stained for microscopic examinations.

### 2.7. Colonization Assay on Suckling Mice Model

The colonizing potential of the mutant IEM101-T was evaluated in the suckling mouse cholera model as previously reported ***(15)***. Postnatal d 5 BALB/c pups were inoculated intragastrically with $1.0 \times 10^7$ CFU of IEM101, IEM101-T, and IEM101-T transformed with pFGY101(IEM101-T/pFGY101). Five mice were used for each strain. After 16 h, mice were sacrificed, and the small intestines were removed, washed three times in PBS, and homogenized in 2 mL of the same buffer. Cell counts were determined by serially diluting and plating of 20-µL droplets in triplicate on LB agar with 50 µg/mL thymidine when required.

## 3. Results

The *thyA* gene was cloned from *V. cholerae* IEM101 as described in **Subheading 2**. Clone pFGY101 was used as template for DNA sequencing. The alignment data showed with that *thyA* gene of IEM101 was completely identical with that of reference strain VCH6514, and interestingly, the start codon also is GTG rather than ATG. The deduced amino acid of *thyA* from IEM101 only shared 32.8% identity with *E. coli*, but to our surprise, plasmid bearing *thyA* gene from *E. coli* was able to complement *thyA* mutant derived from IEM101 (Yu et al., unpublished data).

The homologous recombination performed in IEM101 to obtain IEM101-T generated a 535-bp internal deletion in its *thyA* coding region, which prevents the synthesis of an active thymidylate synthase. Consequently, IEM101-T is impaired in the ability to produce dTTP, an essential precursor of DNA biosynthesis. The deletion was verified with PCR (not shown) and Southern blot (**Fig. 2**). Aside from the morphology change in minimal medium, IEM101-T was indistinguishable by Gram staining and stereotyping from its parent stain IEM101 in complete medium (LB supplemented with thymidine at 200 µg/mL).

In contrast, in LB broth with thymidine less than 5 µg/mL IEM101-T grew at a much lower rate and stopped growing eventually at 1.0 to 2.0 units of absorbance at 600 nm.

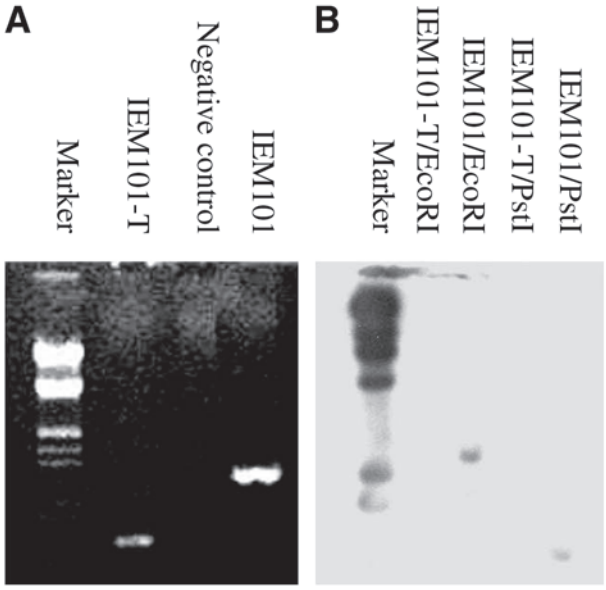


Fig. 2. *thyA* deletion was determined by PCR and Southern blot analysis. *Eco*RI- or *Pst*I-digested genomic DNAs of IEM101 or IEM101-T were probed with Dig-labeled *Bgl*II–*Sty*I *thyA* probe. Arrowheads indicated the hybridized bands on IEM101 genomes, but they were not detectable on its counterpart IEM101-T because of deletion. DNA mass ladders were probed with Dig-labeled *Hind*III-digested πDNAs. PCR was performed using P1 and P2 primers, which spanned the coding sequence of the *thyA* gene. A smaller fragment was obtained from the mutant duo to the internal deletion.

Similar growth curves were obtained for IEM101 in LB and IEM101T in LB supplemented with 50 µg/mL thymidine (data not shown).

However, in minimal medium, the mutation introduced in *thyA* generated a nutritional requirement for thymidine, which could be eliminated by transformation with plasmid pFGY101, containing *thyA* coding fragment. In thymidine-limiting conditions, IEM101-T was a suspension with fine aggregates, whereas IEM101 was evenly turbid. In contrast to IEM101, IEM101-T acquired an elongated filamentous appearance in LB supplemented with thymidine at 50 µg/mL or less (**Fig. 3**).

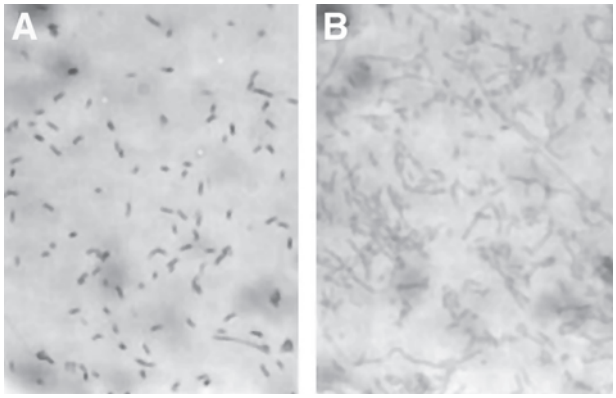


Fig. 3. Morphology of wild-type *V. cholerae* IEM101 **(A)** and mutant IEM101-T **(B)**. Light microscopy revealed that IEM101-T growing in LB supplemented with thymidine at 50 µg/mL changed into elongated filament-shaped phenotype, whereas IEM101 remained unchanged. (Viewed under 10 × 100 magnification.)

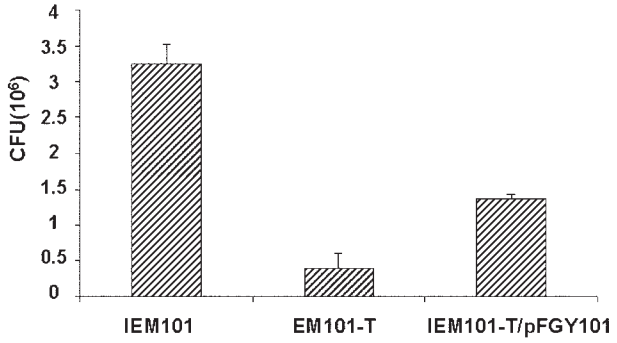


Fig. 4. Colonization ability of IEM101-T compared to IEM101 and IEM101-T transformed with pFGY101. Each value represents the average for five mice in a single experiment. The standard deviations are represented by error bars.

IEM101-T was able to colonize the intestine of suckling mice, but the recovered vibirios was 10-fold less than that of IEM101 (**Fig. 4**). We also found that IEM101T/pFGY101 colonized in the suckling mouse cholera model as well as IEM101, indicating that as a selection marker, *thyA* could prevent loss of plasmid during intestinal colonization.

## 4. Discussion

In this study, the *thyA* gene of *V. cholerae* IEM101 was cloned, sequenced, and mutated by homologous recombination. Except for 10-fold reduced colonizing potential, IEM101-T showed equivalent phenotypes and growth properties with IEM101.The relevance of *thyA* mutation for colonization defect were also reported previously, and this question is still debatable ***(7,8,16)***. Although IEM101-T displayed slightly reduced colonizing potential, it was not at the expense of its immunogenicity, and this was demonstrated in our later studies on IEM108, which was derived from IEM101-T by introducing *ctxB* and *rstR* with a chromosome-plasmid lethal balanced system. The vibriocidal antibody response in sera from rabbits immunized with IEM101 and IEM108 were found to ber very similar ***(17)***.

Compared with those spontaneous mutants selected by trimethoprim resistance, mutant IEM101-T was a genetically defined *thyA* mutant generated by homologous recombination, in which one one-third of the *thyA* was deleted from its genome. Mutant generated in this manner would be incapable of bearing unexpected mutation or reversion, which had been shown by work of Kaper and many others ***(12,13,18)***. Just like with attenuated vaccines for other diseases, biological containment is an important issue in the development of live cholera vaccines. Thymidine auxotrophy has been proposed as an environmental biosafe feature for environmentally released vaccines, as free pyrimidines are scare in natural ecosystems ***(9)***. Without significant alterations of its features as a vaccine candidate, IEM101-T is improved over its parental strain IEM101 by the enhancement of the environmental safety.

The selective adherence of *V. cholerae* to the M cells of the gastrointestinal tract makes it a potent stimulus to the common mucosal immune system ***(19,20)***. IEM101 has been proved to be safe, able to colonize the intestinal mucosa, and to induce a strong immune response in terms of IgA, IgG, and IgM and vibriocidal antibodies ***(10)***. IEM101 has been used as a carrier for ex-

pression of detoxified derivatives of cholera toxin and heterologous antigens such as fragment C from tetanus toxin (TetC) and tracheal colonization factor from *Bordetella pertussis* (Tcf) ***(21,22)***. Therefore IEM101-T can be further developed into a very useful lethal balanced system for the delivery of foreign genes by using *thyA* locus instead of antibiotics as markers for maintenance and selection of plasmids.

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## References

1. Tauxe, R., Seminario, L., Taipia, R., and Libel, M. O. (1994) The Latin American epidemic. In: Vibrio cholerae *and Cholera: Molecular to Global Perspectives* (Wachsmuth, I. K., Blake, P. A., and Olsvik, Ø. eds.). ASM Press, Washington, DC, pp. 321–344.
2. Zhang, D., Xu, Z., Sun, W., and Karaolis, D. K. (2003) The vibrio pathogenicity island-encoded mop protein modulates the pathogenesis and reactogenicity of epidemic *Vibrio cholerae*. *Infect. Immunol.* **71**, 510–515.
3. Mekalanos, J., Goldberg, I., Miller, V., et al. (1985) Genetic construction of cholera vaccine prototypes. In: Lerner, R., Chanock, R., and Brown, F., eds. Vaccines 85, Cold Spring Harbor, pp. 101–105.
4. Okada, T., Yanagisawa, K., and Ryan, F. J. (1960) Elective production of thymine-less mutants. *Nature* **188,** 340–341.
5. Farmer, J. L. and Rothman, F. (1965) Transformable thymine-requiring mutant of *Bacillus subtilis*. *J. Bacteriol.* **89,** 262–263.
6. Ross, P., O'Gara, F., and Condon, S. (1990) Cloning and characterization of the thymidylate synthase gene from *Lactococcus lactis* subsp. *lactis*. *Appl. Environ. Microbiol.* **56,** 2156–2163.
7. Ross, P., O'Gara, F., and Condon, S. (1990) Thymidylate synthetase gene from *Lactococcus lactis* as a genetic marker: an alternative to antibiotic resistance genes. *Appl. Environ. Microbiol.* **56,** 2164–2169.
8. Levine, M. M., Kaper, J. B., Herrington, D., et al. (1988) Volunteer studies of deletion mutants of *Vibrio cholerae* O1 prepared by recombinant techniques. *Infect. Immun.* **56**, 161–167.
9. Valle, E., Ledon, T., Cedre, B., et al. (2000) Construction and characterization of a nonproliferative El Tor cholera vaccine candidate derived from strain 638. *Infect. Immun.* **68**, 6411–6418.
10. Liu, Y. Q., Qi, G. M., Wang, S. X., et al. (1995) A natural vaccine candidate strain against cholera. *Biomed. Environ. Sci.* **8**, 350–358.
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual, 2nd ed.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
12. Michalski, J., Galen, J. E., Fasano, A., and Kaper, J. B. (1993) CVD110, an attenuated *Vibrio cholerae* O1 El Tor live oral vaccine strain. *Infect. Immunol.* **61**, 4462–4468.
13. Hone, D. M., Harris, A. M., Chatfield, S., Dougan, G., and Levine, M. M. (1991) Construction of genetically defined double aro mutants of *Salmonella typhi*. *Vaccine* **9**, 810–816.
14. Tison, D., Murray, P. R., and Baron, E. J. (eds.) (1999) *Manual of Clinical Microbiology*. Washington, DC, ASM Press, pp. 497–506.
15. Cedré, B., L. García, L., García, H., Fariñas, M., Talavera, A., and Infante, J. F. (1998) Intestinal colonization of the infant mouse model by attenuated and virulent *Vibrio cholerae* strains. *Arch. Med. Res.* **29**, 231–234.
16. Attridge, S. R. (1995) Thymine auxotrophy as an attenuating marker in *Vibrio cholerae*. *Microb. Pathog.* **19**, 11–18.
17. Liang, W., Wang, S., Yu, F., et al. (2003) Construction and evaluation of a safe, live, oral *Vibrio cholerae* vaccine candidate, IEM108. *Infect. Immun.* **71**, 5498–5504.
18. Miller, V. L. and Mekalanos J. J. (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requries *toxR*. *J. Bacteriol.* **170,** 2575–2583.
19. Owen, R. L., Pierce, N. F., Apple, R. T., and Cray, W. C. Jr. (1996) M cell transport of *Vibrio cholerae* from the intestinal lumen into Peyer's patches: a mechanism for antigen sampling and for microbial transepithelial migration. *J. Infect. Dis.* **153**, 1108–1118.
20. Winner, L. 3rd, Mack, J., Weltzin, R., Mekalanos, J. J., Kraehenbuhl, J. P., and Neutra, M. R. (1991) New model for analysis of mucosal immunity: intestinal secretion of specific monoclonal immunoglobulin A from hybridoma tumors protects against *Vibrio cholerae* infection. *Infect. Immun.* **59**, 977–982.
21. Chen, I., Finn, T. M., Yanqing, L., Guoming, Q., Rappuoli, R., and Pizza, M. (1998) A recombinant live attenuated strain of *Vibrio cholerae* induces immunity against tetanus toxin and *Bordetella pertussis* tracheal colonization factor. *Infect. Immun.* **66**, 1648–1653.
22. Fontana, M. R., Monaci, E., Yanqing, L., et al. (2001) IEM101, a naturally attenuated *Vibrio cholerae* strain as carrier for genetically detoxified derivatives of cholera toxin. *Vaccine* **19**, 75–85.