S2-4

ANALYSIS AND MANIPULATION OF CANDIDATE GENES FOR DIARRHEAL DISEASE VACCINE DEVELOPMENTS

Kim, Young-Chang

School of Life Sciences, Chungbuk National University

ABSTRACT

Diarrheal diseases are a major cause of both illness and death in developing countries and are caused by rotavirus, *Shigella* spp., *Salmonella* spp., enterotoxigenic *Escherichia coli* (ETEC), and *Vibrio* spp.

In this study, for the development of vaccine against diarrheal diseases caused by *Shigella sonnei, Salmonella typhimurium, E. coli* O157, and *Vibrio cholerae*, cloning and nucleotide sequence analysis of genes and characteristics of their gene products in *E. coli* were performed.

For construction of attenuated strain of *S. sonnei* KNIH104 and *Salmonella typhimurium* KNIH100, the *aroA* genes were cloned, respectively. The recombinant plasmid pJP⊿A45 containing *aroA* deleted region and suicide vector (pJP5603) was constructed. The *aroA* gene deleted mutants were constructed using this recombinant plasmid.

For cloning gene encoding antigenic region of *E. coli* O157 KNIH317, the O-antigen synthesis gene cluster and *slt* gene was cloned. The *E. coli* XL1-Blue cells harboring this recombinant plasmid showed cytotoxicity in Vero cells.

The *ctx* gene was cloned for the purpose of antigenic region against *V. cholerae* KNIH002. Sequence analysis confirmed that the virulence gene cassette was consisted of *ace, zot, ctxA* and *ctxB* genes.

I. INTRODUCTION

Among the infectious and parasitic diseases occurring in the world, diarrheal diseases are the third most common infectious illness (The World Health Report 1999, http://www.who.int/whr/1999/en/disease.htm). The numbers of death caused by diarrheal disease were 2.2 million in 1998. This mortality occupied 4.1% of the total death (53.9 million) in the world.

Diarrheal diseases are a major cause of both illness and death in developing countries and are caused by rotavirus, *Shigella* spp., *Salmonella* spp., enterotoxigenic *Escherichia coli* (ETEC), and *Vibrio* spp. Specially, these diseases are very serious in young children.

Also, diarrheal diseases by *Shigella* spp., pathogenic *E. coli*, and *Vibrio* spp. occurred continuously in Korea. Therefore, the bacterial dysentery, typhoid fever, and cholera are classified as class I national communicable diseases in Korea.

Ⅱ. Nucleotide sequences analysis of *aroA, asd,* and *recA* genes from *S. sonnei* KNIH104 and *S. typhimurium* KNIH100

Shigella spp. and *E. coli* are very closely related in evolutional steps. On the basis of the Kohara map of *E. coli* K12, it was predicted that the *asd* gene (*recA* gene) of *S. sonnei* KNIH104 may be located in 1.7-kb *Bam*HI fragment (2.8-kb *Kpn*I fragment) of the chromosome. These prediction were confirmed in this study. The *aroA, asd,* and *recA* genes from *S. sonnei* were cloned and sequenced for the first time in this study.

(1) The *aroA* gene

According to analysis by Blast E-mail server, nucleotide (amino acid) sequences of the *aroA* gene of *S. sonnei* KNIH104 (GenBank accession No. AF101225) exhibited 98.5(99) %, 99.5(99) %, 79.2(94) %, and 79.1(94) % identity with those of *S. dysenteriae* (GenBank accession No. U82268), *E. coli* (GenBank accession No. X00557), *Sal. typhi* (GenBank accession No. X54545), and *Sal. typhimurium* (GenBank accession No. Y10355), respectively. Also, the *serC* and *aroA* genes of *S. sonnei* lie in a single operonic structure like those of *E. coli, S. dysenteriae, Sal. typhi,* and *Sal. typhimurium.*

The *aroA* gene from *Sal. typhimurium* KNIH100 was composed of 1,284 base pairs with ATG initiation codon and TAA termination codon. Sequence comparison of the *aroA* gene exhibited 99 %, 98 %, and 77 % identity with those of *S. typhi* Ty2, *S. typhimurium,* and *E. coli* respectively. As in the cases of *Shigella sonnei* and *E. coli*, the *serC* and *aroA* genes lie in a single operonic structure.

(2) The *asd* gene

Nucleotide (amino acid) sequences of the *asd* gene of *S. sonnei* KNIH104 (GenBank accession No. AF101226) exhibited 99.0(100) %, 85.3(98) %, and 46.4(55) % identity with those

of *E. coli* (GenBank accession No. V00262), *Sal. typhimurium* (GenBank accession No. AF015781), and *V. cholerae* (GenBank accession No. Y15281), respectively (Fig. 30).

In comparison of the length of Asd polypeptide, the length of Asd polypeptide of *S. sonnei* and *E. coli* was same. But, the length on the site of N-terminal region in Asd polypeptide of *Sal. typhimurium* was more larger (23 amino acids) than those of *S. sonnei* and *E. coli.*

(3) The *recA* gene

The *recA* gene of *S. sonnei* KNIH104 (GenBank accession No. AF101227) was highly related to that of *E. coli* (GenBank accession No. X55552) and *S. flexneri* (GenBank accession No. X55553), being 99.5% and 99.7% identity, respectively (Fig. 31).

The amino acid sequences of RecA polypeptide from *E. coli* and *S. flexneri* have 100% identity. But, the RecA polypeptide of *S. sonnei* KNIH104 was different only one site. Arg-86 site of RecA polypeptide presented in *E. coli* and *S. flexneri* was replaced by Leu in *S. sonnei* KNIH104. In the Block database, Lys (73%), Arg (15.9%), Ala (7.3%), Gln (2.4%) and Ser (1.2%) were found at this site among 82 sequenced RecA proteins. This data indicated that Arg-86 site was not strictly conserved.

Ⅲ. Nucleotide sequence analysis of the *slt* gene from *E. coli* O157 KNIH317 and verocytotoxic activity of its gene products in *E. coli*

(1) The *slt* gene

Nucleotide (amino acid) sequences of the *sltA* gene of *E. coli* O157 KNIH317 (GenBank accession No. AF175707) exhibited 100(100) %, 99.3(99) %, 95.9(99) %, and 99.5(99) % identity with those of the *slt*IIA gene of *E. coli* bacteriophage 933W (GenBank accession No. X07865), the variant *sltA* gene of *E. coli* (GenBank accession No. L11079), the *sltA* gene of *E. coli* (GenBank accession No. L11078), and the *sltIIA* gene of *E. coli* (GenBank accession No. Z37725), respectively (Fig. 32). Also, nucleotide (amino acid) sequence of the *sltB* gene of *E. coli* O157 KNIH317 (GenBank accession No. AF175707) exhibited 100(100) %, 95.6(97) %, 86.3(92) %, and 99.3(98) % identity those of the *sltIIB* gene of *E. coli* bacteriophage 933W (GenBank accession No. X07865), the variant *sltB* gene of *E. coli* (GenBank accession No. L11079), the *sltB* gene of *E. coli* (GenBank accession No. L11078), and the *slt*IIB gene of *E. coli* (GenBank accession No. Z37725), respectively (Fig. 33).

From these above results, it can be concluded that the *slt* gene cloned from *E. coli* O157

KNIH317 belongs to *slt*II family and the strain used in this study may be lysogeny of *E. coli* bacteriophage 933W. However, the cloned *sltII* gene differs to the *sltII* of 933W in three sites of *sltII* upstream region and four sites of *sltII* downstream region (Fig. 34). From this analysis, it was concluded that clonal turnover might occur during the lysogenic cycle or transfer to each other's host cells.

(2) The verocytotoxic activity

The *E. coli* XL1-Blue cells harboring the recombinant plasmids (pOVT45 or pOVT45-1) encoding shiga-like toxin had almost same toxicity in Vero cells. From these results, it was confirmed that the produced shiga-like toxins were presented in host cells.

Ⅳ. Nucleotide sequence analysis of the virulence gene cassette from *V. cholerae* KNIH002 and cytotoxic activity of its gene products in *E. coli*

(1) The *ace* and *zot* genes

By nucleotide sequence analysis of the cloned virulence gene cassette, the last four nucleotides of *ace* gene (A TGA) including the stop codon (TGA) was also the first nucleotides of *zot* gene (ATG A). Nucleotide sequence of the *ace* gene of *V. cholerae* KNIH002 (GenBank accession No. AF175708) exhibited 100 % identity with that of *V. cholera* E7946 El Tor Ogawa strains (GenBank accession No. Z22569) deposited unique in the GenBank (Fig. 35). And, nucleotide (amino acid) sequence of the *zot* gene exhibited 100(100) % and 98.9(99) % identity with those of *V. cholerae* O139-Tor Ogawa strain (GenBank accession No. AF123049) and *V. cholerae* 395 Classical Ogawa strains (GenBank accession No. M83563), respectively (Fig. 36). Specially, the Ile-45, Ala-100, Ala-272, and Ala-281 sites of Zot polypeptide presented in *V. cholerae* 395 Classical Ogawa strain was replaced by Met-45, Ala-100, Ala-272, and Ala-281 in *V. cholerae* KNIH002. But, all of these amino acids belong to the nonpolar (hydrophobic) R group.

(2) The *ctxAB* gene

The last four nucleotides of *ctxA* gene (A TGA) including the stop codon (TGA) was also the first nucleotides of *ctxB* gene (ATG A). Nucleotide (amino acid) sequence of the *ctxA* gene of *V. cholerae* KNIH002 (GenBank accession No. AF175708) exhibited 99.7(100) %, 99.6(99.6) %, 99.6(99.6) % identity with those of *V. cholerae* O139-Bengal 1854 strain

(GenBank accession No. D30053), *V. cholerae* S7 strain (GenBank accession No. D30052), and *V. cholerae* 2125 strain (GenBank accession No. X00171), respectively (Fig 37). The Ser-46 site of CtxA polypeptide presented in *V. cholerae* KNIH002 strain was replaced by Asp-46 in *V. cholerae* S7 strain. But, this amino acid belong to the polar uncharged R group. And the Met-213 site of CtxA polypeptide presented in *V. cholerae* KNIH002 strain was replaced by Ile-213 in *V. cholerae* 2125 strain. But, this amino acid belong to the nonpolar hydrophobic R group.

Nucleotide (amino acid) sequence of the *ctxB* gene of *V. cholerae* KNIH002 (GenBank accession No. AF175708) exhibited 99.2(97.6) %, 99.2(97.6) %, 98.4(96) % identity with those of *V. cholerae* O139-Bengal 1854 strain (GenBank accession No. D30053), *V. cholerae* S7 strain (GenBank accession No. D30052), and *V. cholerae* 2125 strain (GenBank accession No. X00171), respectively.

(3) The cytotoxic activity

Whereas the cholera toxin molecules in *E. coli* was confined to the periplasm, either toxin or their respective B subunit pentamers were translocated through the outer membrane of *V. cholerae* into the extracellular medium. As in other bacteria, where several functions were required for the extracellular transport of proteins, secretion of cholera toxin in *V. cholerae* was affected by several genes (*epsE*) essential for the extracellular secretion of cholera toxin. In this study, the used toxins were crude extract prepared by sonication. Therefore, the *E. coli* XL1-Blue cells harboring the recombinant plasmids (pCTX75-1, or pCTX7504-1) encoding cholera toxin A-subunit had the toxicity in CHO cells. From these results, the cholera toxins produced in *E. coli* were presented (remained) in the cells because *E. coli* has not genes for secretion of cholera toxin. Also, *V. cholerae* KNIH002 indicate very weakly toxicity in CHO cells. This indicates that most of the cholera toxins produced in *V. cholerae* were secreted into the medium.

V. Utility of the constructed balanced-lethal vector

Plasmid stability in host cell is very important in the construction of avirulent live vaccine strains carrying foreign antigens. Nakayama *et al.*, (1988) developed a balanced-lethal host-vector system that imposes a requirement in all living bacteria to maintain the recombinant plasmids. The system uses a plasmid that carries the *asd* gene of *Streptococcus*

mutans as the only selectable marker in combination with a *Salmonella* strain with a chromosomal deletion in its own *asd* gene. This imposes a requirement for the bacteria to maintain the plasmids since in the absence of DAP in the medium, *asd* mutants undergo lysis.

The aims of these studies were to clone the *asd* gene of *S. sonnei* for use it in the Asd⁺ vector/Δ*asd* host system, and to constructed balanced-lethal vector (pSKA47 and pSKA47A) using the cloned *asd* gene and pBluescrip SK(+) vector. These vector will be used usefully for expression of foreign genes for polyvalent vaccine in the avirulent vaccine strain (Δ*asd* host).

Ⅵ. Utility of the *aroA* deleted mutants for vaccine against shigellosis

The construction of *aroA* gene deleted mutants of *S. sonnei* KNIH104 (104-7A, 104-7B, and 104-7C) was described. These mutants in *aroA* gene have been fully defined at the molecular level by PCR technique and Southern hybridization. But, during this studies, *aroA* deletion mutants were lost a large plasmid encoding genes essential for virulence and antigenic region. Therefore, the large plasmid must be introduce into these auxotroph by electroporation, conjugation or any other methods. These attenuated auxotrophic mutants may be used usefully as a vaccine against *S. sonnei* isolated in Korea.

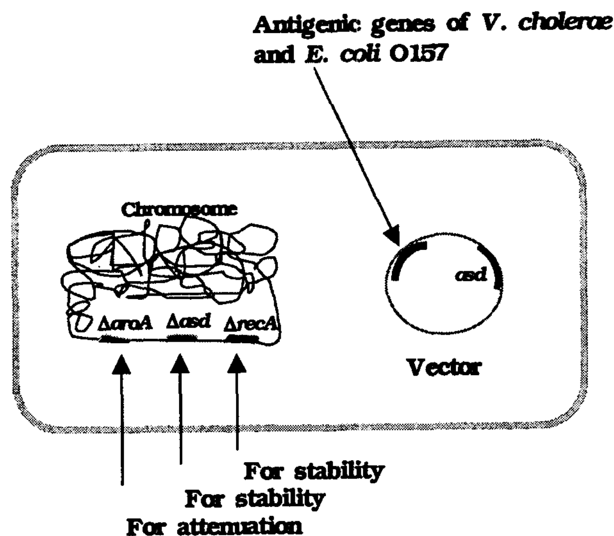
Ⅶ. The prospect of vaccine development against diarrheal diseases

For construction of vaccine against shigellosis, the *aroA, asd,* and *recA* genes were cloned for construction of auxotroph, construction of balanced-lethal vector, and stability of vector from *S. sonnei* KNIH104, respectively. And, the genetically defined *aroA* deletion mutants of *S. sonnei* KNIH104 were constructed. Also, the *slt*II gene and virulence gene cassette (*ace, zot,* and *ctxAB* genes) were cloned from *E. coli* O157 KNIH317 and *V. cholerae* KNIH002 isolated in Korea, respectively.

Now, the triple mutants (Δ*aroA*Δ*asd*Δ*recA*) are being constructed using the *aroA* deleted auxotroph for attenuation, balanced-lethal vector system, and stability of the vector. And, the expression of antigenicity region in the constructed balanced-lethal vector are being studied using *sltIIB* gene encoding shiga-like toxin B-subunit of *E. coli* O157 and *ctxB* gene encoding cholera toxin B-subunit of *V. cholerae,* respectively.

If the above described researches are successful executed, the constructed vaccine may be

used usefully as a trivalent vaccine against *S. sonnei, E. coli* O157, and *V. cholerae.*



Strategy for construction of trivalent vaccine against *S. sonnei, V. cholerae* and *E. coli* O157.

REFERENCES

1. **Lee, K.U., Y.R. Ju, Y.M.A. Park. K.J. Lee, Y.J. Lee, C.K. Kim. S.G. Hong, Y.C. Park, S.Y. Hwang, J.Y. Lim, and Y.C. Kim.** 1996. Construction of allelic exchanged mutants for a gene replacement of *Shigella sonnei. J. Korean Soc. Microbiol.* **31**: 35-43.

2. **Park, Y.C., H.J. Shin, and Y.C. Kim. 1999a. Cloning and nucleotide sequence analysis of the *asd* gene from *Shigella sonnei*** KNIH104S. ***Korean J. Microbiol.*** **35: 13-17.**

3. **Park, Y.C., H.J. Shin, and Y.C. Kim.** 1999b. Cloning and nucleotide sequence analysis of the *recA* gene from *Shigella sonnei* KNIH104S isolated in Korea. *J. Biochem. Mol. Biol.* 32: 436-439.

4. **Park, Y.C., H.J. Shin, and Y.C. Kim.** 1999c. Cloning and nucleotide sequence analysis of

verotoxin gene from *Escherichia coli* O157 KNIH317 isolated in Korea. *J. Microbiol.* 37; 168-174.

5. **Park, Y.C., H.J. Shin, S.G. Hong, S.Y. Hwang, G.D. Park, Y.R. Ju, K.J. Lee, and Y.C. Kim**. 1997. Cloning and nucleotide sequence of the *aroA* gene from *Shigella sonnei* KNIH104S. *J. Korean Soc. Microbiol.* **32**: 151-159.

6. **Park, Y.C., S.Y. Hwang, H.J. Shin, K.J. Lee, Y.R. Ju, Y.S. Bae, and Y.C. Kim.** 1998. Construction of genetically defined *aroA* deletion mutants of *Shigella sonnei* KNIH104S. *J. Korean Soc. Microbiol.* **33**: 373-380.

7. **Shin, H.J., Y.C. Park, and Y.C. Kim**. 1999. Cloning and nucleotide sequence analysis of the virulence gene cassette from *Vibrio cholerae* KNIH002 isolated in Korea. *The Korean J. Microbiol.* **35**: 205-210.