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The Bacterial Surface Expression of SARS Viral Epitope using *Salmonella typhi* Cytolysin A

Hong Hua Piao[1], Jihyoun Seong[1], Man Ki Song[2], Youn Uck Kim[3], Dong-Jun Shin[1], Hyon E Choy[1] and Yeongjin Hong[1*]

[1]Clinical Vaccine R&D Center and Department of Microbiology, Chonnam National University Medical School, Gwangju, Korea; [2]International Vaccine Institute, Seoul, Korea; [3]Sun Moon University, A-San, Korea

The cytolysin A (ClyA) is a 34 kDa pore-forming cytotoxic protein and expressed by some enteric bacteria including *Salmonella typhi*. This toxin is transported on the bacterial surface and secreted without posttranslational modification. Using the surface display of ClyA, the expression vectors for 193-aa immunogenic antigen of spike protein (termed S1E) from severe acute respiratory syndrome coronavirus (SARS-CoV) were constructed. The vectors carried a gene encoding *S. typhi* ClyA conjugated to S1E at the C terminus (termed ClyA-S1E) and *asd* gene in pGEM-T and pBR322, named pGApLCS1E and pBApLCS1E, respectively. An *asd*-mutated *E. coli* transformed with these vectors could grow without diaminopimelic acid (DAP), indicating that they were stably maintained in such mutants. ClyA-S1E recombinant proteins from these vectors were expressed on the surface of the attenuated *S. typhimurium* deficient of global virulence gene regulator, ppGpp. However, they did not show the hemolytic activity on the blood agar plate and cytotoxicity against HeLa cells. To examine whether bacteria expressing ClyA-S1E induced the immune response against S1E, *S. typhimurium* deficient of ppGpp and Asd was transformed with these vectors and orally immunized in mice. In the western blotting against GST-conjugated S1E using the immunized mouse sera, it was shown that the significant band was detected in the mouse serum by the bacteria transformed with pGApLCS1E but not with pBApLCS1E. It indicates that the immune response producing antibody was dependent on the expression level of ClyA-S1E. Therefore, ClyA delivery system can be used for SARS vaccine development.

Key Words: Surface display, Cytolysin A, SARS

INTRODUCTION

Many *Salmonella* attenuated strains have been developed as vaccines against the infectious diseases by the pathogenic *Salmonella* spp. such as food poisoning, typhoid fever and so on (1) and as antigen delivery system for the immunogenic antigens from bacteria, virus, fungi and mammalian (2). To obtain high immunogenic response against the delivered antigens, it has been known that the antigens should be expressed on the bacterial surface or be secreted (3). The surface display technology such as *Pseudomonas syringae* ice nucleation protein (4), HlyA *E. coli* secretion system (5) and *Salmonella enterica* MisL autotransporter (6) have been developed up.

The *clyA/hlyE/sheA* genes have been identified in *E. coli* and *Salmonella entritica* serovar Typhi and Paratyphi A and encode 34 kDa proteins with over 90% amino acid identity (7). The structure of ClyA consisting of an elaborated helical

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*Corresponding author: Yeongjin Hong. Department of Microbiology, Chonnam National University Medical School, 5 Hak-1-dong, Dong-ku, Gwangju 501-746, Korea.
Phone: +82-62-220-4138, Fax: +82-62-228-7294
e-mail: yjhong@chonnam.ac.kr
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bundle some 100 Å long has been clearly defined using X-ray crystallography (7). These proteins are transported on the bacterial surface without any posttranslational modification and aggregate to form lipid-associated oligomers with N- and C-termini exposure (8). Such vesicle-mediated export and assembly of ClyA were demonstrated, and the resulting outer membrane vesicles were larger than normally formed vesicles (8). The activities transporting to the bacterial surface and aggregating in secretary vesicles raise the possibility that the immunogenic antigens conjugated to ClyA would be efficiently transported on the bacterial surface. For example, *E. coli* ClyA conjugated to β-lactamase at N- or C-terminus has been efficiently exported to the extracellular medium (9). Recently, the attenuated *Salmonella* transformed with *clyA* gene fused with *Bacillus anthracis* antigen enhanced immnunogenicity against intranasally immunized mice (10).

The spike protein of severe acute respiratory syndrome coronavirus (SARS-CoV) can be divided into putative extracellular S1 and transmembrane/intracellular S2 domains (11). The S1 and S2 domains are responsible for receptor binding and membrane fusion, respectively (11). Through deletion mutant analysis, 193-aa epitope (residues 318~510) in S1 (termed S1E in this work) has been identified as the binding domain for angiotensin converting enzyme 2 (ACE-2), a receptor of S protein (12, 13). S1E domain could be the main target for the development of SARS-CoV vaccine (14). The human IgG1 Fc recombinant protein conjugated to S1E induced highly potent antibody responses against S1 protein in the immunized rabbits and their serum completely inhibited SARS-CoV infection (15).

In this study, we constructed the expression vectors carrying two genes, *clyA* for surface delivery of S1E epitope and *asd* for stable plasmid maintenance, and the expression and surface display of the recombinant S1E conjugated to ClyA (termed ClyA-S1E) were analyzed on the attenuated *S. typhimurium* strain after transformation by vectors. Furthermore, it was examined whether the bacteria transformed with these vectors could induce the immune response producing anti-S1E antibody in orally immunized mice.

MATERIALS AND METHODS

Construction of plasmids

To construct the expression vectors for ClyA, its gene was amplified by PCR with primers (9), sal6 (5'-CGGTACCGATATCACCGATG) and sal1 (5'-CTCGTCAGCCCGGTAACGAC) using the *S. typhi* genomic DNA as a template and directly cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) behind the *lac* promoter, named pGCSO. To construct the ClyA expression vector without a stop codon, PCR was done with pGCSO using primers, sal6 and sal7 (5'-GAAAATACTAGTGACGTCAGGAACCTCGAAAAG). The PCR product was digested with *Cla*I and *Spe*I, and the 200 bp fragment of 3' region was ligated into the corresponding sites of pGCSO, generating pGCSX.

The DNA fragment encoding S1E epitope was amplified by PCR with primers, SARS6 (5'-TCCGTGGAATTCACTAGTAACATCACCAACCTGTG) and SARS7 (5'-GCTCAGATGCATCTCGAGTCACACGGTGGCGGGCGCGT) using pcDNA3.1-SARS S1 (a gift of Dr. Michael Farzan) as a template (12). The amplified PCR fragment was digested with SpeI and NsiI and cloned into the same sites of pGCSX. The fragment carrying *asd* gene was amplified by PCR with primers, ASD1 (5'-CGCGCAGGGCCCGCACATCTCTTTGCAGGAAAA) and ASD2 (5'-CTGCAAGCATGCCTACGCCAACTGGCGCAGCAT) using *S. typhimurium* genomic DNA as a template (16). The resulting 1.1 kb PCR fragment was digested with *Apa*I and *Sph*I and cloned into the same sites of pGCSX. The plasmid named pGApLCS1E. *Apa*I-*Nsi*I fragment carrying a gene encoding ClyA-S1E and *asd* gene from pGApLCS1E was cloned into the same site of pBR322, named pBApLCS1E. The simple maps of these two plasmids were depicted in Fig. 1.

To construct the expression vector for ClyA, the fragment was amplified by PCR with primers, Sal12 (*Bam*HI) (5'-GAAGGATCCATGACCGGAATATTTGCAGAA) and Sal11 (*Xho*I) (5'-CTCCTCGAGGACGTCAGACGTCAGGAACCTC), using pGCSO as a template. The resulting PCR fragment was digested with *Bam*HI and *Xho*I and

cloned into the same sites of pGEX-4T-1 (Amersham, Pittsburgh, PA, USA), named pGEX-ClyA. To construct the expression vector for S1E epitope, the fragment was amplified with primers, SARS6 (*Eco*RI) (5'-GAAGAATTCAACATCACCAACCTGTG) and SARS7 (*Xho*I) (5'-CTCCTCGAGCACGGTGGCGGGCGCG) using pcDNA-SARS S1 as a template. The resulting PCR fragment was digested with *Eco*RI and *Xho*I and cloned into the same sites of pGEX-4T-1, named pGEX-S1E.

Plasmids used in this work were described in Table 1.

Bacteria strains and growth conditions

Wild-type *S. typhimurium* (ATCC 14028s) was used as the parent strain for subsequent genetic modifications. SHJ2037 (*relA::cat*, *spoT::kan*), a ppGpp-deficient *S. typhimurium* strain, was previously described (17). SHJ2107 (*relA::cat*, *spoT::kan*, *asd::kan*) was the *asd* mutant of SHJ2037. All bacteria strains except the *asd* mutants were grown aerobically at 37℃ in LB broth. The asd mutants were grown aerobically at 37℃ in LB broth containing diaminopimelic acid (DAP, 100 μg/ml). Bacteria strains used in this work were described in Table 1.

Preparation of rabbit anti-ClyA and anti-S1E antisera

E. coli BL21(DE3) codon plus RP transformed with pGEX-ClyA or pGEX-S1E were prepared after 0.5 mM IPTG induction. Cell pellet was dissolved in 1 volume of 2× sample buffer (100 mM Tris-HCl, pH 6.8/20% glycerol/ 10% SDS) and boiled. The proteins were separated by SDS-PAGE. The predominant 60 kDa of GST-tagged ClyA and 46 kDa of GST-tagged S1E protein bands were excised from the gel after cold KCl stain (0.25 M KCl/1 mM DTT) and grinded in nitrogen gas. The grinded gel slices were dissolved in PBS (500 μl) and mixed with the same volume of complete Freund's adjuvant (Sigma, ST. Louis, MO, USA). The mixture was immunized subcutaneously. Three and six weeks later, the rabbit was immunized twice more by the mixture of the grinded gel slice and incomplete

Table 1. Bacterial strains and plasmids used in this study

Strain or Plasmids	Description	Source
SCH2005	Wild-type *S. typhimurium*	ATCC14028s
SHJ2037	*spoT::cat, relA::kan*	Lab stock
SHJ2107	*spoT::cat, relA::kan, asd::kan*	Lab stock
SCH2008	*osmZ::Tn10*	Lab stock
HJ1019	*asd::kan*	Lab stock
pUC19	Cloning vector	Promega
pGEM-T easy	Cloning vector	Promega
pGEX-4T-1	Cloning vector	Amersham
pBR322	Cloning vector	Promega
pGEX-ClyA	pGEX-4T-1 carrying 1.4 kb *clyA* PCR fragment	This study
pGEX-S1E	pGEX-4T-1 carrying 579 bp *S1E* PCR fragment	This study
pUCLYA	pUC19 carrying 1.4 kb PCR *clyA* fragment	This study
pGCSO	pGEM-T easy vector carrying 1.4 kb *clyA* PCR fragment with a stop codon	This study
pGCSX	pGEM-T easy vector carrying 1.4 kb *clyA* PCR fragment without a stop codon	This study
pGA	pGApLCS1E deleting *clyA-S1E* genes	This study
pGApLC	pGApLCS1E deleting *S1E* gene	This study
pGApLCS1E	pGEM-T easy vector with *asd*, *clyA-S1E* genes	This study
pBApLCS1E	pBR322 with *asd*, *clyA-S1E* genes	This study

Freund's adjuvant. The rabbit serum obtained after the third injection was used as anti-ClyA and anti-S1E antisera.

Western blot analysis

After overnight culture, 100 μl of boiled bacterial lysate was separated in 10% SDS-PAGE gel and subsequently transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was incubated with 1:250 diluted rabbit anti-ClyA or anti-S1E antisera at room temperature for 2 h and washed. After then, it was incubated for 1.5 h at room temperature with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Accurate Chemical, Westbury, NY, USA). After final washing, bound antibody was detected with an FAST^{TM} BCIP/NBT (Sigma).

Fluorescence microscopy

Bacteria were cultured overnight and fresh cultured for 6 h with 2.5% dilution in LB medium. The culture (400 μl) was centrifuged at 13,000 rpm for 1 min, and washed three times with 1× PBS. Bacterial pellet was resuspended in 4% formaldehyde (200 μl) and mounted on Lab-TecII chamber Slide (Nunc Inc, Naperville, IL, USA). After incubation for 20 min at room temperature, slide was washed by 1× PBS. Then, slide was incubated in 1× PBS containing 1% BSA for 30 min at room temperature. After washing three times by 1× PBS, bacteria were stained with rabbit anti-ClyA antiserum and FITC-conjugated anti-rabbit IgG (Sigma). The stained bacteria were observed by fluorescence microscope.

Acetone protein precipitation

Bacteria were cultured overnight and centrifuged at 6,000 rpm for 10 min. Three milliliters supernatant were mixed with 9 ml cold acetone and kept on ice for 15 min, then centrifuged at 6,000 rpm for 30 min. After drying completely at room temperature for 10 min, the pellet was dissolved in 2× SDS loading dye (20 μl) for 5 min at the 50℃. Then, after boiling for 5 min, the pellet was separated on 10% SDS-PAGE gel and western blot assay was performed by polyclonal anti-ClyA and anti-S1E rabbit antisera.

Hemolytic activity assay

Bacteria were cultured overnight and diluted to 2% in 1× PBS. After bacterial streaking on 5% sheep blood agar plate, bacteria were cultured for 2 days in a CO_2 incubator. The bacteria with hemolytic activity showed white haloes around colonies.

Cytotoxicity assays using HeLa cells

After culture for 4 h in fresh LB medium, bacteria were washed in 1× PBS. After the bacteria infection (MOI=100) against HeLa cells (2×10^6) for 9 h, the cells were observed by microscopy. The dead cells showed the aberrant shrunk cell morphology. Then cells were washed by 1× PBS and detached with trypsin-EDTA. The cells were resuspended in 300 μl HMW buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA) and lyzed by the addition of 5 μl proteinase K (10 mg/ml) and 300 μl 0.2% SDS-HMW buffer. After phenol:chloroform treatment, genomic DNA was precipitated in 0.7 volume of isopropanol. Genomic DNA was dissolved by TE buffer after 70% ethanol washing. Finally, DNA was separated in 2% agarose gel. The dead cells by apoptosis showed DNA ladder.

Immunization

BALB/c female mice (Damul Animal Laboratory Center, Cheongju, Korea) used in this work were eight weeks old. Mice were housed in the animal chamber maintained at 22 to 23℃ with 12 h of illumination every day. Prior to oral immunization, mice were allowed to access to water but fasted overnight. Food and water were returned 12 h after oral immunization. Mice were randomly divided into 4 groups with 10 mice per group. Group 1 was not infected by bacteria. The other groups were orally immunized with 5×10^8 CFU bacteria resuspended in 0.3 ml of sterile PBS. The blood was obtained by cardiac puncture from mice after 4 weeks and centrifuged at 13,000 × g for 5 min at 4℃ to obtain serum. The existence of antibody in serum was detected by western blotting against GST-tagged S1E protein. Briefly, lysates of *E. coli* transformed with pGEX-S1E were separated by SDS-PAGE, blotted on a nitro-

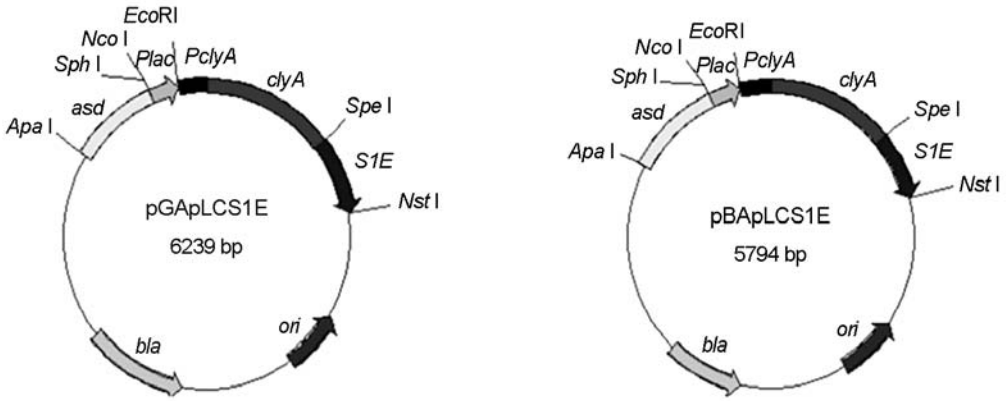

Figure 1. Construction of plasmids. pGApLCS1E and pBApLCS1E were constructed from pGEM-T and pBR322, respectively and carried both of *clyA-S1E* and *asd* genes. The *clyA-S1E* genes are induced by *lac* promoters.

cellulose membrane and stained with Ponceau S dye. The membrane was cut to the pieces containing single lane. After removing dye, each membrane piece was stained with serum from the immunized mice and AP-conjugated goat anti-mouse IgG. All experiments using mice were followed the guideline approved by the Committee for the Care and Use of Laboratory Animals at Chonnam National University, Gwangju, Korea.

RESULTS

Stable maintenance of vectors carrying *asd* gene

Aspartate β-semialdehyde dehydrogenase (Asd) is an enzyme at the upstream step in lysine and peptidoglycan biosynthesis and essential for the growth (18). Therefore, the plasmids with *asd* gene stably maintained without other selectable marker gene in its mutated bacteria. DAP, a downstream substance in lysine pathway, should be required in a medium to grow *asd*-mutated bacteria (19).

In this work, we constructed two vectors expressing ClyA-S1E, pGApLCS1E and pBApLCS1E, which possessed high and low copy numbers (Fig. 1). These carried *asd* genes to be stably maintained in *asd*-mutated bacteria. The *asd*-mutated *E. coli* (HJ1019) and *S. typhimurium* (SHJ2107) were only grown in the presence of plasmids carrying *asd* gene (data not shown).

Surface display and secretion of ClyA-S1E expressed in *S. typhimurium*

To investigate whether ClyA-S1E protein could be expressed in *S. typhimurium*, the vectors expressing ClyA-S1E were transformed into SHJ2037 strain which is completely deficient of ppGpp and avirulent against mice (20). Whole lysates after boiling were transferred to nitrocellulose membrane and stained with anti-ClyA and anti-S1E antisera (Fig. 2A). In western blotting using anti-ClyA antiserum (left panel), a 34-kDa protein band was detected in bacteria transformed with pGCSO carrying *clyA* (lane 2). In contrast, bacteria transformed with pGApLCS1E and pBApLCS1E showed 53-kDa protein bands (lanes 3 and 4). These proteins should be ClyA-S1E because the bands of the same size were detected in the western blotting by anti-S1E antiserum (right panel). The band intensity of ClyA-S1E expressed by pGApLCS1E was higher than that of pBApLCS1E. It indicates that the expression of ClyA-S1E is dependent upon the copy number of the plasmid.

ClyA is displayed on the bacterial surface and secreted (8). We examined whether ClyA-S1E was expressed on the surface of *S. typhimurium* (Fig. 2B). SHJ2037 strains transformed with the indicated vectors were stained with DAPI to stain bacterial DNA (left panels) and anti-ClyA antiserum (right panels). The transformed bacteria carrying pGCSO but not mock vector could be stained with anti-ClyA antiserum (panels 1 *vs*. 2). Bacteria carrying pGApLCS1E and

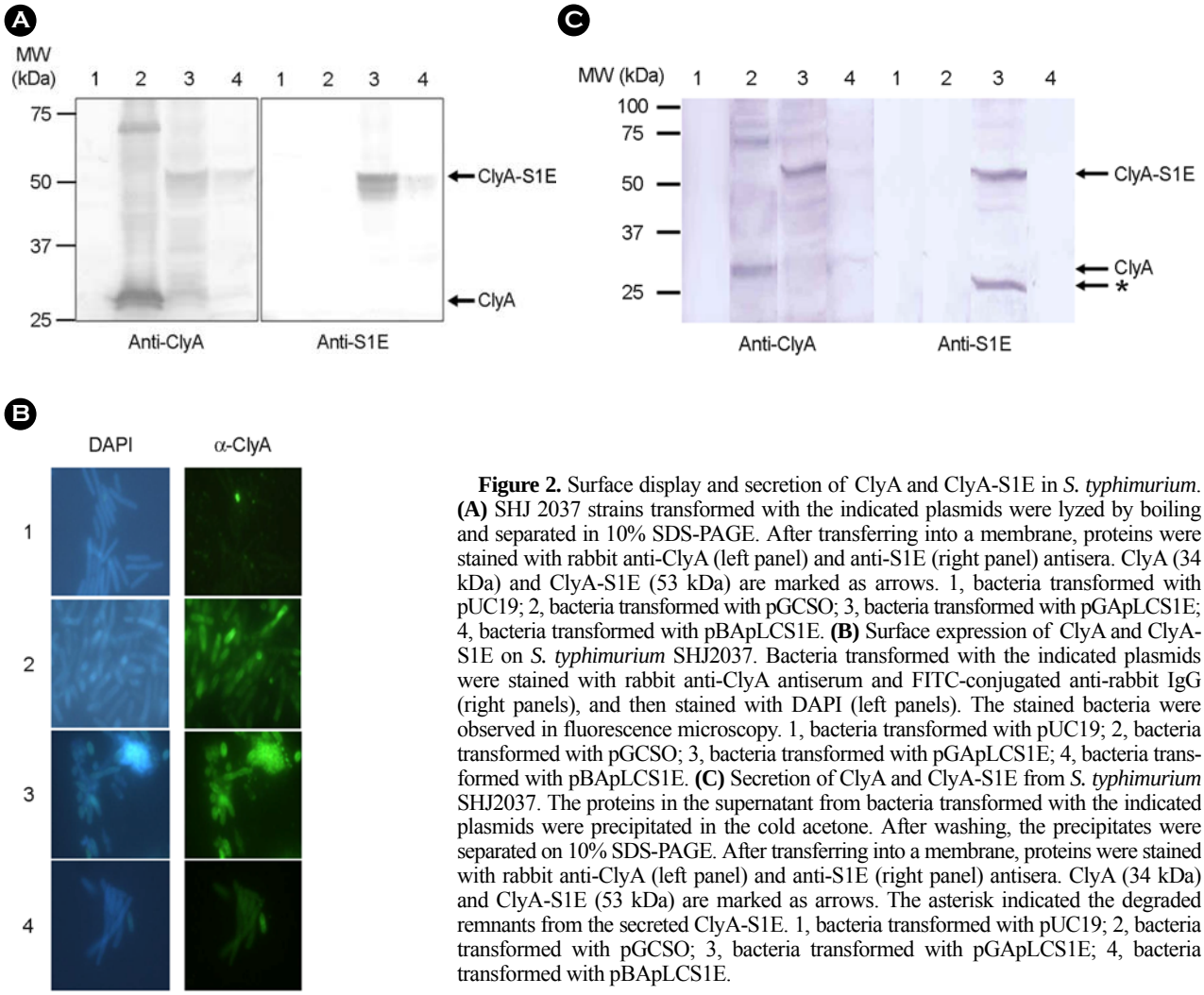

Figure 2. Surface display and secretion of ClyA and ClyA-S1E in *S. typhimurium*. **(A)** SHJ 2037 strains transformed with the indicated plasmids were lyzed by boiling and separated in 10% SDS-PAGE. After transferring into a membrane, proteins were stained with rabbit anti-ClyA (left panel) and anti-S1E (right panel) antisera. ClyA (34 kDa) and ClyA-S1E (53 kDa) are marked as arrows. 1, bacteria transformed with pUC19; 2, bacteria transformed with pGCSO; 3, bacteria transformed with pGApLCS1E; 4, bacteria transformed with pBApLCS1E. **(B)** Surface expression of ClyA and ClyA-S1E on *S. typhimurium* SHJ2037. Bacteria transformed with the indicated plasmids were stained with rabbit anti-ClyA antiserum and FITC-conjugated anti-rabbit IgG (right panels), and then stained with DAPI (left panels). The stained bacteria were observed in fluorescence microscopy. 1, bacteria transformed with pUC19; 2, bacteria transformed with pGCSO; 3, bacteria transformed with pGApLCS1E; 4, bacteria transformed with pBApLCS1E. **(C)** Secretion of ClyA and ClyA-S1E from *S. typhimurium* SHJ2037. The proteins in the supernatant from bacteria transformed with the indicated plasmids were precipitated in the cold acetone. After washing, the precipitates were separated on 10% SDS-PAGE. After transferring into a membrane, proteins were stained with rabbit anti-ClyA (left panel) and anti-S1E (right panel) antisera. ClyA (34 kDa) and ClyA-S1E (53 kDa) are marked as arrows. The asterisk indicated the degraded remnants from the secreted ClyA-S1E. 1, bacteria transformed with pUC19; 2, bacteria transformed with pGCSO; 3, bacteria transformed with pGApLCS1E; 4, bacteria transformed with pBApLCS1E.

pBApLCS1E also were stained with anti-ClyA antiserum (panels 3 and 4), indicating that ClyA-S1E proteins were delivered and exposed on the surface in the transformed bacteria.

Next, we examined whether ClyA-S1E was secreted from *S. typhimurium* (Fig. 2C). After the same transformed bacteria in Fig. 2B were cultured in LB media, proteins in the culture supernatants were precipitated by acetone. The precipitated proteins were separated in SDS-PAGE and western-blotted by anti-ClyA (left panel) or anti-S1E (right panel) antisera. ClyA and ClyA-S1E proteins were detected in the supernatant of bacteria transformed with pGCSO and pGApLCS1E, respectively (lanes 2 and 3). However, we hardly detected ClyA-S1E in the supernatant of bacteria transformed with pBApLCS1E. It indicates that high expression of ClyA-S1E is required for its efficient secretion.

Hemolytic and cytotoxic activities of ClyA-S1E expressed in *S. typhimurium*

ClyA is a poreforming toxin to induce hemolysis against red blood cells and cytotoxicity against various cells (9). To examine whether ClyA-S1E recombinant protein still possessed the hemolytic activity, SHJ2037 bacteria transformed with ClyA-S1E expression vectors were cultured on the sheep blood plate for 2 days in 5% CO_2 (Fig. 3A, left panels). Bacteria carrying pGCSO showed strong hemolytic haloes. However, bacteria with pGApLCS1E or pBApLCS1E hardly did. It indicates that ClyA-S1E possesses very weak

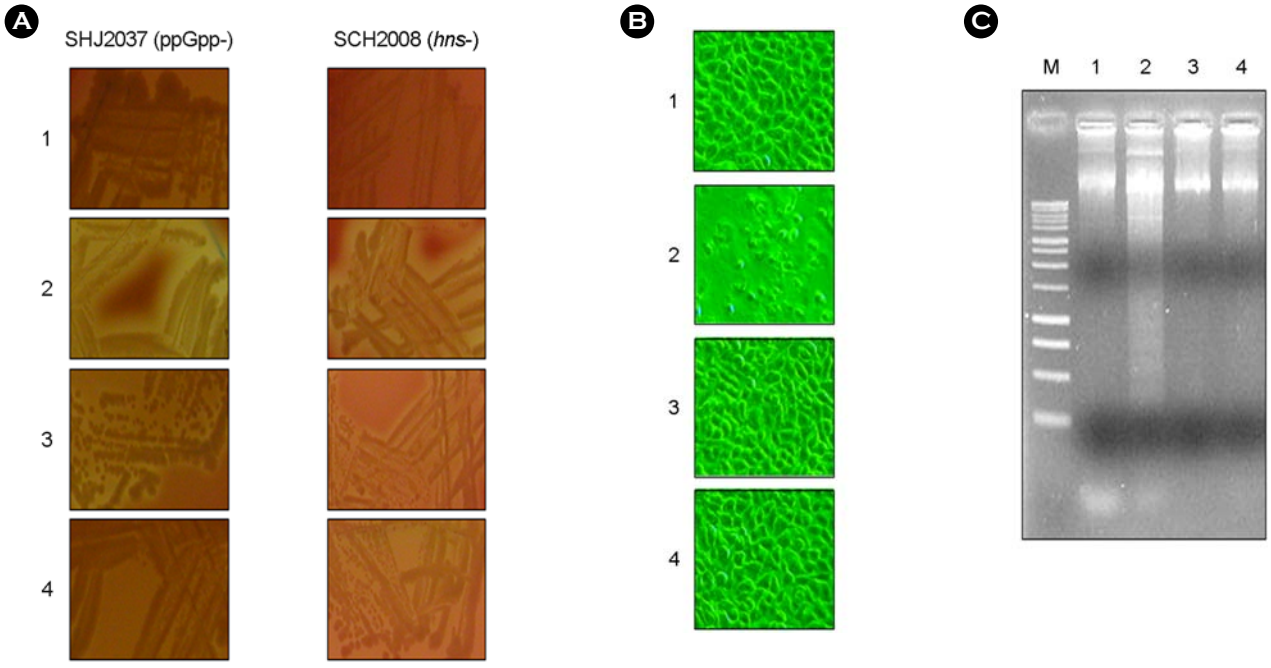

Figure 3. ClyA-S1E lost hemolytic and cytotoxic acitivities. **(A)** Hemolysis assay of *S. typhimurium* strains expressing the ClyA and ClyA-S1E proteins. ppGpp-deficient SHJ2037 (left panels) and hns-mutated SCH2008 with (right panels) transformed with the indicated plasmids were cultivated on the sheep blood agar plate for 2 days. 1, bacteria transformed with pUC19; 2, bacteria transformed with pGCSO; 3, bacteria transformed with pGApLCS1E; 4, bacteria transformed with pBApLCS1E. **(B)** The cytotoxicity of *S. typhimurium* expressing ClyA and ClyA-S1E against HeLa cells. The cells (2×10^6) were cultured for 9 hr after inoculation of *S. typhimurium* SHJ2037 transformed with the indicated plasmids (2×10^8). Cell morphology was observed with microscopy. 1, bacteria transformed with pUC19; 2, bacteria transformed with pGCSO; 3, bacteria transformed with pGApLCS1E; 4, bacteria transformed with pBApLCS1E. **(C)** The genomic DNA fragmentation by apoptosis was assayed in 2% agarose gel electrophoresis using the same cells in (B).

hemolytic activity, compared to ClyA.

The *clyA* gene has been strongly expressed in *hns*-mutant *S. typhi* as HNS is the transcription factor to repress *clyA* promoter (21). As the *clyA* gene used in this work contained its promoter region, there was the possibility that ClyA-S1E would be more expressed and secreted in the absence of HNS, causing more hemolysis. It was tested using *hns*-mutated *S. typhimurium* strain, SCH2008 (right panels). Similarly to the result in SHJ2037, the hemolysis in SCH-2008 transformed with pGApLCS1E or pBApLCS1E was hardly detected. It indicates that *clyA* promoter does not work for the expression of ClyA and ClyA-S1E in the vectors.

Next, we examined cytotoxicity of ClyA-S1E against HeLa cells. SHJ2037 transformed with the indicated vectors was inoculated in HeLa cells and the cell death was observed by microscopy after 9 h (Fig. 3B). Consistent with a previous report (8), the host cells with aberrant shrunk morphology were induced by the bacteria carrying *clyA* gene caused (panel 2). However, any bacteria expressing ClyA-S1E did not show cell death at all in this condition (panels 3 and 4). ClyA induces apoptosis as well as necrosis (22). Apoptosis causes DNA fragmentation in the genome (15) and is induced by *Salmonella* infection (23). Using the same HeLa cells in Fig. 3B, host genomic DNA fragmentation caused by apoptosis was analyzed (Fig. 3C). Only HeLa cells inoculated by bacteria carrying only *clyA* gene showed DNA ladder in genomic DNA (lane 2). These results indicate that ClyA-S1E does not induce the cell death by necrosis and apoptosis.

Anti-S1E antibody detection in mice orally immunized by *S. typhimurium* expressing ClyA-S1E

Finally, we examined whether *S. typhimurium* expressing ClyA-S1E could enhance the production of anti-S1E antibody in mice. SHJ2107 bacteria, deficient of ppGpp and

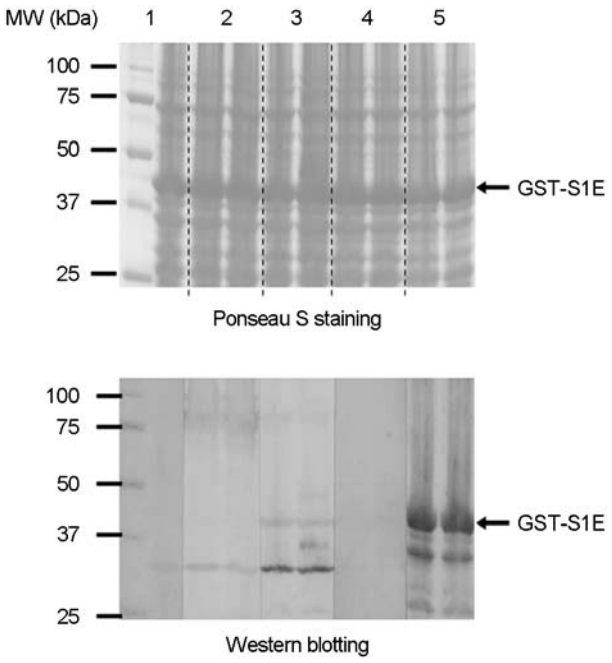

Figure 4. Detection of anti-S1E antibody in mice orally immunized by *S. typhimurium* expressing ClyA-S1E. *S. typhimurium* SHJ2107 strains carrying the deficiency of ppGpp and Asd were transformed with the indicated plasmids. BALB/C mice were orally immunized by the transformed bacteria (5×10^8) and the blood was taken from mice at day 30. The serum was used for western blotting against *E. coli* lysates expressing GST-conjugated S1E (upper panel). Ponseau S staining of *E. coli* lysates expressing GST-conjugated S1E separated in SDS-PAGE (lower panel). Western blotting against *E. coli* lysates containing GST-tagged S1E using the immunized antiserum. As a control, the same sample was western blotted by rabbit anti-S1E antiserum. 1, mouse antiserum after the infection by bacteria with pGA; 2, mouse antiserum after the infection by bacteria with pGApLC; 3, mouse antiserum after the infection by bacteria with pGApLCS1E; 4, mouse antiserum after the infection by bacteria with pBApLCS1E; 5, rabbit anti-S1E antiserum.

Asd, were transformed with pGApLCS1E and pBApLCS1E (Fig. 4). As controls, the same bacteria were transformed with pGA carrying only *asd* gene or pGApLC carrying both of *asd* and *clyA* genes. After mice were orally immunized by the transformed bacteria for 30 days, the presence of anti-S1E antibody in the mouse serum was examined using the western blotting against GST-conjugated S1E. The same amount of *E. coli* lysates expressing GST-S1E were loaded in each lane of SDS-PAGE and separated. In the transferred membrane, 45-kDa GST-S1E proteins strongly stained with Ponceau S dye (upper panel). In the western blotting analysis against membrane strips containing two lanes (lower panel), the immunized serum by bacteria with pGApLCS1E but not pBApLCS1E detected GST-S1E with the significant intensity (lanes 3 and 4), which was the same size of the band by rabbit anti-S1E antiserum. It indicates that *S. typhimurium* highly expressing ClyA-S1E could induce the production of anti-S1E antibody in orally immunized mice.

DISCUSSION

ClyA found in *S. typhi* and some enterobacteria including *E. coli* is exposed on the bacterial surface and secreted as a monomer or oligomeric assembly (21). There were trials to deliver the antigen using surface display and secretion of ClyA (9, 10). In this work, 193-aa S1E epitope from SARS-CoV S protein was chosen as a cargo antigen because this has been well characterized as a receptor binding domain (22) and vaccine candidate (14). The recombinant protein ClyA-S1E nearly lost cytotoxicity against the host cells, although it was expressed on the bacterial surface. It would be ascribed to the conjugated S1E hindering the normal conformation of monomeric ClyA or the conformational change to make oligomeric assembly. This phenomenon shown in ClyA-S1E is not universal for all ClyAs conjugated to cargo antigen because it was efficiently exported to the extracellular medium and possessed hemolytic activity in the conjugation with β-lactamase (1). Probably, surface or secretion efficiency of the recombinant ClyA protein should be dependent upon the molecular character of the cargo antigens.

ClyA-S1E was expressed more highly in pGApLCS1E than pBApLCS1E, indicating that the expression of antigen is dependent on the gene copy number. Only the detectable level of anti-S1E antibody in the mouse serum orally immunized by the attenuated *S. typhimurium* carrying pGApLCS1E was due to the difference in the expression. Therefore, the increase of ClyA-S1E expression would display the more efficient immune response in vaccine trial using the attenuated *S. typhimurium* expressing ClyA-S1E. To do this, *lac* promoter would be replaced by the more strong promoters such as *tac17* to express ClyA-S1E (23). Nevertheless, the attenuated *S. typhimurium* carrying

pGApLCS1E is anticipated to be used as a live vaccine against SARS-CoV.

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