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Molecular Cloning and Characterization of *clyA* Genes in Various Serotypes of *Salmonella enterica*

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Cytolysin A (ClyA) is a pore-forming hemolytic protein encoded by the *clyA* gene. It has been identified in *Salmonella enterica* serovars Typhi and Paratyphi A. To identify and characterize the *clyA* genes in various *Salmonella enterica* strains, 21 different serotypes of strains isolated from clinical specimens were presently examined. Full-length *clyA* genes were found in *S. enterica* serovar Brandenburg, Indiana, Panama, and Schwarzengrund strains by polymerase chain reaction amplification. The ClyA proteins from these four strains showed >97% amino acid identity to that of *S. enterica* serovar Typhi. Although all four serovars expressed detectable levels of ClyA as determined by Western blot analysis, they did not show a strong hemolytic effect on blood agar, indicating that ClyA may not be efficiently expressed or secreted. *Escherichia coli* transformed with *clyA* genes from the four serovars enhanced production of ClyA proteins and hemolytic activities to a level similar to *S. enterica* serovar Typhi ClyA. The present results suggest that ClyA may play a role in the pathogenesis of *S. enterica* serovar Brandenburg, Indiana, Panama and Schwarzengrund.

***Keywords*:** ClyA, cytolysin, pore-forming toxin, *Salmonella*

Pathogenic bacteria produce many substances that are directly or indirectly toxic to host cells and play a central role in the pathogenesis of infectious disease (Finlay and Falkow, 1997). ClyA (also called HlyE or SheA), a 34-kDa protein first identified in *Escherichia coli*, kills host cells by forming pores in target membranes (Oscarsson *et al*., 1996; del Castillo *et al*., 1997; Ludwig *et al*., 1999; Oscarsson *et al*., 1999; Wallace *et al*., 2000). It is also an important virulence factor in extraintestinal *E. coli* infection. In many cases, *E. coli* is cytotoxic to cultured macrophages due primarily to ClyA (Chen *et al*., 1996; Wai *et al*., 2003).

Salmonella enterica infects a wide range of different hosts and causes a broad spectrum of diseases from gastroenteritis to bacteremia, as well as life-threatening conditions such as typhoid fever (Ohl and Miller, 2001). Genome sequence analysis of *S. enterica* serovar Typhi was revealed in an open reading frame highly homologous to *E. coli clyA* (Parkhill *et al*., 2001). Oscarsson *et al*. (2002) reported that *clyA* is conserved in the human-specific *S. enterica* serovars Typhi and Paratyphi A while the intact *clyA* gene is apparently absent in many other *S. enterica* serovars, including Typhimurium.

More than 2,500 *Salmonella* serotypes have been described. *Salmonella* infection continues to be a major public health problem in Korea (Cho *et al*., 2008). In spite of the recent increase in human nontyphoidal *Salmonella* infections, the presence and expression of ClyA in various *S. enterica* strains has been rarely investigated. In this study, *clyA* genes in 21 different *S. enterica* serotypes isolated from clinical specimens were analyzed.

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Materials and Methods

Bacterial strains and culture media

Twenty-one *S. enterica* serotypes isolated from 115 clinical specimens of diarrheal patients were collected from 2001-2004 in the Gwangju area of Korea (Table 1). *S. enterica* serovar Typhimurium ATCC 14028s and Typhi Ty2 were used as a negative and positive control, respectively. For DNA manipulations, *E. coli* DH5α was used for the gene cloning. The bacterial strains were grown at 37°C in Luria-Bertani (LB) broth or LB supplemented with 1.5% (w/v) agar unless otherwise indicated. The blood agar plates containing 2.3% (w/v) sheep blood were used in the hemolysis test.

Polymerase chain reaction (PCR) and sequence analysis of *clyA* genes

Gene cloning and manipulation were followed as previously described (Sambrook *et al*., 1989). The 1.4-kb *clyA* gene from each strain was amplified by PCR (30 sec at 94°C, 45 sec at 57°C, and 1 min at 72°C) with 35 cycles. The PCR primers, sal1 (5′-CTCGTCAGCCCGGTAAC GAC) and sal6 (5′-CGGTACCGATATCACCGATG) were designed following the gene sequence from *S. enterica* serovar Typhi (Oscarsson *et al*., 2002). PCR products were separated by 1% agarose gel electrophoresis. After purifying the PCR fragments, sequencing was done using an ABI 3100 sequencer (Applied Biosystems, USA) at the Korea Basic Science Institute, Gwangju city, Korea. Putative protein sequences were analyzed using the BLAST algorithm and DNAsis program (HITACHI, Japan).

Gene cloning

The *clyA* genes from *S. enterica* serovar Typhi, Brandenburg, Indiana, Panama, and Schwarzengrund, were termed *clyA*-Typhi, *clyA*-Bran,

Table 1. Detection of *clyA* gene by PCR for *S. enterica* clinical isolates of 21 different serovars

Serotype	No. of tested	No. of positives
Agona(O:B)	1	0
Albert	1	0
Bardo	4	0
Blockley(O:C2)	1	0
Braenderup	4	0
Brandenburg	1	1
Derby	1	0
Enteritidis	34	0
Essen(O:B)	1	0
Hadar	4	0
Indiana	1	1
Kedougou(O:G)	1	0
London	8	0
Mbandaka	1	0
Panama	2	2
Paratyphi B	4	0
Potsdam	4	0
Rissen	3	0
Schleissheim	1	0
Schwarzengrund	1	1
Typhimurium	37	0
Total	115	5

clyA-Indi, *clyA*-Pana, and *clyA*-Schw, respectively. The recombinant plasmids were transformed into *E. coli* DH5α competent cells by an established heat shock method. A *clyA*-Typhi DNA fragment was amplified with sal1 and sal6 primers using *S. enterica* serovar Typhi Ty2 genomic DNA as a template and directly cloned into the pGEM-T easy vector (Promega, USA) behind the *lac* promoter, and was designated pGCSO. To construct the expression vector for ClyA, the DNA fragment was amplified with primers sal12(*Bam*HI) (5′-GAA GGATCCATGACCGGAATATTTGCAGAA-3′) and sal11(*Xho*I) (5′-CTCCTCGAGGACGTCAGACGTCAGGAACCTC-3′) using pGCSO as a template (the restriction endonuclease sites are underlined). The purified PCR product was digested with *Bam*HI and *Xho*I, and the resultant DNA fragment was cloned into the high expression vector pGEX-4T-1 (Amersham, USA). The *clyA* gene fragments of other *S. enterica* serotypes were amplified from their genomic DNA using sal1 and sal6 primers and cloned into pGEM-T easy vector.

Preparation of rabbit anti-ClyA

ClyA was overexpressed in *E. coli* BL21 from plasmid pGEX-ClyA Typhi and culture solution induced with 0.5 mM isopropylthio-galactoside (IPTG), and the cell pellet was supplemented with 1 volume of 2× sample buffer containing 100 mM Tris-HCl, pH 6.8, 20% glycerol, and 10% sodium dodecyl sulfate (SDS). After boiling, the samples were separated by 12% SDS-polyacrylamide gel electrophoresis. The predominant 60-kDa of GST-tagged ClyA protein band was excised from the gel, ground in nitrogen gas, and individually dissolved in 500 μl of phosphate buffered saline (PBS) and the same volume of complete Freund's adjuvant. The mixture was used for the subcutaneous immunization of rabbits (Damul, Korea); after 2 and 4 weeks, each rabbit was immunized twice more by amixture of the ground gel slice and incomplete Freund's adjuvant. Two weeks after the last vaccination, each rabbit was exsanguinated, serum was collected by centrifugation, and the antiserum was absorbed against heat-killed *E. coli* BL21 cells.

Western blotting

To analyze ClyA protein expression, bacteria were grown in LB broth at 37°C with aeration and harvested after 16 h. Cells were thoroughly washed three times with sterile PBS. After boiling for 5 min and centrifugation for 10 min at 12,000×g, supernatants were mixed and separated with 2× SDS-PAGE sample buffer containing 0.125 M Tris, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.1% bromophenol blue. Rabbit anti-ClyA antiserum (1:30,000) and anti-rabbit lgG antiserum was sequentially used for Western blotting.

Nucleotide sequence accession numbers

The nucleotide sequences of *clyA* from *S. enterica* serovar Brandenburg, Indiana, Panama and Schwarzengrund have been submitted to the GenBank database (http://www.ncbi.nlm.nih.gov) (accession nos. FJ205475-FJ205478).

Results

Analysis of *clyA* gene in *S. enterica* serovars

Clinical isolates (n=115) of 21 *S. enterica* serotypes were analyzed by PCR to verify the presence of *clyA* gene, using primers designed based on the gene of *S. enterica* serovar Typhi. Four *S. enterica* serovars, Brandenburg (n=1), Indiana (n=1), Panama (n=2), and Schwarzengrund (n=1) harbored a 1.4-kb fragment that was the same size as that in *S. enterica* serovar Typhi. No similar PCR product was obtained from the other isolates (Table 1 and Fig. 1). Nucleotide sequences from each of the genes were determined and the putative protein sequences showed >97% amino acid identities against ClyA-Typhi proteins. The flanking region sequences of their open reading frames were very similar to that of *clyA*-Typhi (data not shown).

Hemolytic activities and expressions of ClyA cytotoxins in *S. enterica* serovar Brandenburg, Indiana, Panama and Schwarzengrund

To examine whether or not the *S. enterica* serovars carrying the *clyA* gene induced hemolysis, the different bacteria were cultured on blood agar containing sheep erythrocytes. Under standard conditions, each strain showed weak hemolytic activity (Fig. 2A). Subsequently, the level of ClyA expression was measured by Western analysis using an anti-ClyA antiserum. The antiserum detected 34-kDa proteins corresponding to ClyA, in cell lysates of all tested *S. enterica* strains (Fig. 3A). These results indicated that *S. enterica* strains carrying *clyA* genes expressed low amount of the protein in standard culture condition.

Expressions of ClyA from serovars Brandenburg, Indiana, Panama, and Schwarzengrund in *E. coli*

The *clyA* genes of the four serovars were cloned into the pGEM-T easy vector. *E. coli* DH5α transformed with plasmids carrying *clyA*-Bran, *clyA*-Indi, *clyA*-Pana, and *clyA*-Schw displayed enhanced hemolytic activities on blood agar plates (Fig. 2B). Western blotting examination showed that the transformed *E. coli* strains exuberantly produced ClyA protein compared to non-transformed control (Fig. 3B).

Fig. 1. PCR analysis of 1.4-kb *clyA* DNA regions of *S. enterica* of various serovars from clinical specimens. The number of each lane corresponds to the list number of Table 1. Lanes: 6, *S. enterica* serovar Brandenburg; 11, *S. enterica* serovar Indiana; 15, *S. enterica* serovar Panama; 20, *S. enterica* serovar Schwarzengrund; 22, *S. enterica* serovar Typhi; 23, *S. enterica* serovar Typhimurium ATCC 14028s.

Discussion

ClyA is an important virulence factor and a potential gene of *Salmonella* pathogenicity island 18 (SPI-18) (Fuentes *et al.*, 2008). Functional *clyA* homologues have been identified in *S. enterica* serovar Typhi and serovar Paratyphi A (Oscarsson *et al*., 2002). In this study, the *clyA* genes of 115 clinically-relevant *Salmonella* strains belonging to 21 different *S. enterica* serovasrs were examined by PCR using primers based on the *S. enterica* serovar Typhi DNA sequence. The examination found that four clinical strains, *S. enterica* serovar Brandenburg, Indiana, Panama and Schwarzengrund also harbored *clyA* genes (Table 1) with >97% amino acid identity to open reading frame of *clyA*-Typhi. The novel *clyA* genes from the four serovars were functional because transformed *E. coli* DH5α carrying each *clyA* gene displayed hemolytic activities on blood agar plates. Thus, the *clyA* genes of each strain encode an active pore-forming cytolysin.

Lipid bilayer experiments and electron microscopic studies have shown that ClyA forms stable pores in target membranes by assembling into ring-shaped toxin oligomers (Ludwig *et al*., 1999; Tzokov *et al*., 2006). This pore-forming activity is

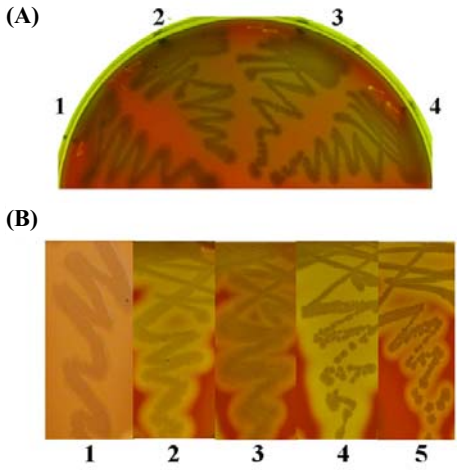

Fig. 2. Hemolytic activity on blood agar of *S. enterica* serovars Brandenburg, Indiana, Panama and Schwarzengrund strains expressing ClyA protein. (A) Hemolysis of wild-type stains. 1, *S. enterica* serovar Brandenburg; 2, *S. enterica* serovar Indiana; 3, *S. enterica* serovar Panama; 4, *S. enterica* serovar Schwarzengrund (B) Hemolysis of *E. coli* DH5α over-expressing *clyA* genes. *E. coli* strains were transformed with pGEM-T easy vector carrying *clyA* genes from four *S. enterica* serovar stains. 1, *E. coli* DH5α; 2, *clyA*-Bran; 3, *clyA*-Indi; 4, *clyA*-Pana; 5, *clyA*-Schw.

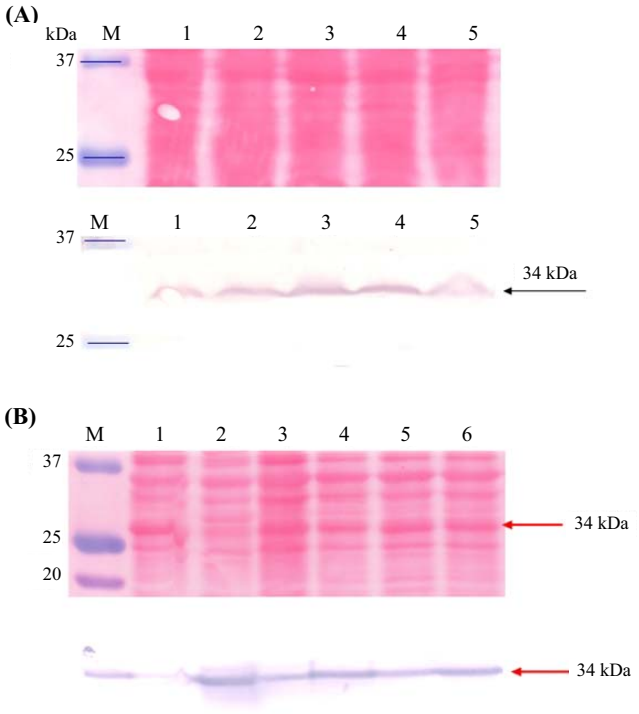

Fig. 3. Western blot analysis of ClyA. (A) Expression of ClyA protein in *S. enterica* wild-type strains. Lanes: 1, *S. enterica* serovar Brandenburg; 2, *S. enterica* serovar Indiana; 3, *S. enterica* serovar Panama; 4, *S. enterica* serovar Schwarzengrund; 5, *S. enterica* serovar Typhi. (B) Expression of ClyA protein in *E. coli* DH5α transformed with *S. enterica* serovar *clyA* genes. Lanes: 1, pUC19; 2, *clyA*-Typhi; 3, *clyA*-Bran; 4, *clyA*-Indi; 5, *clyA*-Pana; 6, *clyA*-Schw.

responsible for lysis of erythrocytes of several mammalian species. Hemolytic toxin belonging to one of several classes of virulence factors contribute to bacterial pathogenicity. The *clyA* gene is under the control of several transcriptional regulators; under standard *in vitro* cultivation conditions, its expression is strongly repressed (von Rhein *et al*., 2006). This is due to repression of *clyA* transcription by the nucleoid protein H-NS (Westermark *et al*., 2000). Nevertheless, the expression of ClyA in *E. coli* can be activated to a level that suffices to evoke a hemolytic phenotype when certain transcriptional regulators, such as SlyA from *E. coli* or *S. enterica* serovar Typhimurium (Ludwig *et al*., 1995; Oscarsson *et al*., 1996; Ludwig *et al*., 1999), MprA (EmrR) from *E. coli* (del Castillo *et al*., 1997), HlyX from *Actinobacillus pleuropneumoniae* (Green and Baldwin, 1997), or FnrP from *Pasteurella haemolytica* (Uhlich *et al*., 1999) are overproduced in this strain. Cui *et al.* (2009) suggested that iron inhibits the expression of ClyA in *S*. Typhi, and that free iron depletion may be one of the causes of *S*. Typhi-mediated induction of ClyA *in vivo*. ClyA expression of *S. enterica* serovar Brandenburg, Indiana, Panama, and Schwarzengrund, like *S. enterica* serovar Typhi, proved to be strongly down-regulated under standard laboratory growth conditions. Thus, when grown in a rich medium, wild-type *S. enterica* serovar Brandenburg, Indiana, Panama and Schwarzengrund produce ClyA only in very small, basal amounts that are detectable only within the bacterial cells, and do not show a *clyA*-dependent hemolytic phenotype (data not shown). Hemolytic activity was absent in the other 17 serotypes, in which *clyA* gene was not detected. Futher detailed *in vitro* analyses will be required to fully analyze the complex relationships between ClyA and hemolysin, and their consequences for *clyA* expression.

As a human and animal pathogen, *Salmonella* species are naturally ubiquitous and display multiple modes of transmission (von Rhein, *et al.*, 2006; Fuentes, *et al.*, 2008). *S. enterica* serovar Brandenburg causes bacteremia (Rodríguez *et al*., 1998). *S. enterica* serovar Indiana causes acute acalculous cholecystitis (Campbell and Eckman, 1975). *S. enterica* serovar Panama contaminates breast milk and caused infants' meningitis (Chen *et al*., 2005). *S. enterica* serovar Schwarzengrund has been the source of the international spread of multidrug-resistance in food products and causes asymptomatic spondylitis (Golding and Robertson, 1985). As all these strains carry the *clyA* gene, the presence of ClyA may be one of the causes of the virulence of *S. enterica* serovar Brandenburg, Indiana, Panama, and Schwarzengrund.

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