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Effect of Iron on Cytolysin A Expression in *Salmonella enterica* serovar Typhi

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Previously, a novel protein ClyA (Cytolysin A) has been identified in *Escherichia coli* K-12, *Salmonella enterica* serovars Typhi and Paratyphi A and *Shigella*. *Salmonella* spp. synthesize substantial amounts of ClyA upon infection of the human host, although the mechanism by which ClyA is induced *in vivo* is unclear. Since environmental signals could control the expression of virulence determinants, ClyA expression in *S.* Typhi Ty2 was tested by Western blotting in the presence of normal pooled human serum (NPS). The level of ClyA expression increased in the presence of NPS in a concentration-dependent manner. RPMI 1640 medium similarly induced ClyA expression. ClyA expression was inversely proportional to the concentration of iron in RPMI medium. Therefore, we speculated that iron inhibited the expression of ClyA in *S.* Typhi Ty2, and free iron depletion may be one of the causes of *S.* Typhi-mediated induction of ClyA *in vivo*. Transcription from a *clyA-lacZ* fusion gene decreased as iron concentration increased, but not as significantly as the ClyA protein expression. It is concluded that the regulatory effect of iron on *clyA* expression is mainly at translational level.

***Keywords*:** cytolysin A (ClyA), iron, *S.* Typhi Ty2

A cryptic gene *clyA* (also called *sheA* or *hlyE*), which encodes a pore-forming protein, was identified first in *Escherichia coli* K-12, and has been the focus of numerous studies due to its hemolytic and cytotoxic activities (Oscarsson *et al.*, 1999). Many reports suggested *clyA* is normally silent, with the hemolytic phenotype emerging either when transcription factors *slyA* and *mprA* are overexpressed (Ludwig *et al.*, 1995; Oscarsson *et al.*, 1996; del Castillo *et al.*, 1997) or when the *hlyE* gene is derepressed in H-NS deficient *E. coli* strains (Westermark *et al.*, 2000). H-NS is a general transcription silencer that represses a large number of genes in Gram-negative bacteria (Stoebel *et al.*, 2008). SlyA, which is an important regulator of genes required for virulence in *Salmonella* (Ellison and Miller, 2006), antagonizes H-NS-mediated repression of *hlyE* transcription in *E. coli* (Wyborn *et al.*, 2004; Lithgow *et al.*, 2007). *clyA* is preserved in human-specific typhoid *Salmonella* Typhi and Paratyphi A (Oscarsson *et al.*, 2002). ClyA protein displays high sequence similarity and similar function with *E. coli* HlyE, and the expression of *clyA* is also activated by the *Salmonella* transcription factor SlyA (Oscarsson *et al.*, 2002; von Rhein *et al.*, 2009). Analysis of several *Shigella* strains revealed that they harbor only nonfunctional *clyA* gene copies (von Rhein *et al.*, 2008). *Salmonella* Typhi *clyA* gene plays a role in invasion of cultured epithelial cells (Fuentes *et al.*, 2008) and is induced significantly during the infection process (von Rhein *et al.*, 2006). It has been suggested that ClyA protein is an important virulence determinant for these *Salmonella* pathogens when they infect human hosts. Apparently, ClyA plays a role in the pathogenesis associated with typhoid and paratyphoid fever.

Environmental cues such as low iron, elevated temperature, osmolarity, pH, oxygen, CO_2, and ions play important roles in controlling the expression of virulence determinants in bacteria (Mekalanos, 1992). Low-iron is considered to be a predominant signal, since iron is needed for important cellular functions including the transport and storage of oxygen, and as a catalyst in electron transport processes (Litwin and Calderwood, 1993). However, free iron in equilibrium with transferrin-bound iron is extremely limited in normal human serum (Bullen *et al.*, 1978); thus, bacteria must take up either free iron from host sources or utilize iron-binding compounds to obtain iron, and these mechanisms are closely linked to bacterial virulence. Hemolysins and siderophores have been implicated as major virulence factors for bacteria to acquire iron from the host. Siderophore, which are specific Fe(III)-binding agents, can sequester and solubilize iron in the environment, and compete effectively with the host iron-binding compounds (Ratledge and Dover, 2000), while hemolysins can increase the level of available iron via lysis of erythrocytes and subsequent release of hemoglobin and heme (Stoebner and Payne, 1988; Ochsner *et al.*, 2000). Therefore, expression of a gene encoding hemolysin increases under the condition of iron limitation (Waalwijk *et al.*, 1983; Griffiths and McClain, 1988; Stoebner and Payne, 1988).

Since iron regulates diverse virulence genes in bacteria, we speculated that ClyA expression would be controlled by

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iron in *Salmonella* Typhi in the iron-deprived conditions prevailing in the human host. We report here that ClyA in *Salmonella enterica* serovar Typhi is induced in iron limiting conditions.

Materials and Methods

Bacteria strains, media, and growth conditions

Salmonella Typhi Ty2 ATCC 19430 was kindly provided by Seoul National University, *Salmonella* Typhimurium ATCC 14028s and *E. coli* DH5α containing plasmid pGCSO that overexpresses *clyA* were used as contrastingly comparative strains. Strain information is provided in Table 1. Generally, bacteria were cultured aerobically at 37°C, or with 100 μg/ml ampicillin (USB, USA) when necessary. Normal pooled human serum (NPS) was obtained from 10 healthy blood donors and was inactivated at 56°C for 30 min. To create low-iron or high-iron growth conditions, Luria-Bertani (LB) broth in the absence or presence of the addition of the iron chelator 2,2'-dipyridyl (final concentration 0.1 mM) and RPMI medium in the absence or presence of 1.2∼24 μM $Fe(NO_3)_3$, $FeCl_3$, and $FeSO_4$ were used.

Preparation of rabbit anti-ClyA antibody

A *clyA* DNA fragment was amplified with primers: sal6; 5'-CGGTACCGATATCACCGATG-3' and sal1; 5'-CTCGTCA GCCCGGTAACGAC-3' using *S*. Typhi genomic DNA as a template and directly cloned into the pGEM-T easy vector (Promega, USA); the resulting plasmid was designated pGCSO. To construct the expression vector for ClyA, the DNA fragment was amplified with primers: sal12; 5'-GAAGGATCCA TGACCGGAATATTTGCAGAA-3' and sal11; 5'-CTCCTC GAGGACGTCAGACGTCAGGAACCTC-3' using pGCSO as a template. The purified polymerase chain reaction (PCR) product was digested with *Bam*HI and *Xho*I, and the resultant DNA fragment was cloned into the GST-fusion expression vector pGEX-4T-1 that had been digested with the same restriction enzymes. The plasmid was designated pGEX-ClyA.

E. coli BL21 transformed with pGEX-ClyA was induced with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG), and cell pellet was supplemented with 1 volume of 2× sample buffer (100 mM Tris-HCl; pH 6.8, 20% glycerol, 10% sodium dodecyl sulfate). After boiling, the samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The predominant 60-kDa of the glutathione-S-transferase (GST)-tagged ClyA protein band was excised from the gel and ground in liquid nitrogen, and the ground gel slices were dissolved in 500 μl phosphate buffered saline (PBS) and the same volume of complete Freund's adjuvant. The mixture was used to immunize a rabbit (obtained from Damul, Korea) subcutaneously after 2 and 4 weeks along with incomplete Freund's adjuvant. Two weeks after the last vaccination, the rabbit was exsanguinated and the serum was collected by centrifugation. The antiserum was absorbed against heat-killed and IPTG-induced *E. coli* BL21 cells harboring pGEX-4T-1.

Western blotting

Strains were grown in LB broth overnight, and then subcultured to stationary phase in various media for 4 or 8 h in the absence or presence NPS or $Fe(NO_3)_3$. Bacteria pellets were resuspended with 1× PBS and diluted to A_{600}=1.0. One hundred microliter of boiling bacterial lysates were then resolved by 12% SDS-PAGE and the electrophoretically resolved proteins were transferred to a nitrocellulose membrane (Bio-Rad, USA). The resulting membrane was incubated with 1:400-diluted rabbit anti-ClyA antiserum at room temperature for 2 h and probed with 1:5000-diluted alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Sigma, USA) as the second antibody at room temperature for 1 h. Finally, AP activity was revealed by using a SIGMA FAST™ 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of STJH1029 was extracted using Trizol reagent (Life Technologies, USA). DNase treatment was performed for 15 min at room temperature to a final volume of 10 μl containing 2 U of amplification grade DNase (Invitrogen, USA). The DNase reaction was terminated by addition of 2.5 mM EDTA, followed by inactivation at 65°C for 15 min. First-strand cDNA was synthesized using SuperScript™

Table 1. Bacteria strains and plasmids used in this study

Strains or plasmids	Description	Reference or source
STJH1029	*Salmonella* Typhi Ty2 (ATCC 19430)	ATCC
SCH2005	*Salmonella* Typhimurium (ATCC 14028s)	ATCC
ECA2007	*E. coli* DH5α containing pGCSO	This work
STJH1106	STJH1029 containing pRPC4151	This work
pGEX-4T-1	Amp^R, expression vector	Amersham
pGEX-ClyA	1.3 kb PCR fragment of clyA with *Bam*HI and *Xho*I cloned into pGEX-4T-1 expression vector	This work
pCR®2.1-TOPO®	Amp^RKm^R, cloning vector	Invitrogen
pRS415	Amp^R, *lacZ* transcription fusion vector	Ratledge and Dover (2000)
pGCSO	1.4 kb PCR fragment of *clyA* with *Nco*I and *Spe*I cloned into pGEM-T easy vector	This work
pTPC2101	405 bp PCR fragment of *clyA* and *t1476* promoter with *Eco*RI and *Bam*HI cloned into pCR®2.1-TOPO®	This work
pRPC4151	405 bp PCR fragment of *clyA* and *t1476* promoter with *Eco*RI and *Bam*HI cloned into pRS415	This work

First-Strand Synthesis System for RT-PCR (Invitrogen). The DNA fragment between *clyA* and *t1476* was generated using the PCR primers: pclyA1; 5'-GCCGAATTCGCAAGCCATT TTGGCTATGAAGC-3' and pclyA2; 5'-GCCGGATCCATC TGCGGTTTCGATCGCGC-3', and the region between genes *t1476* and *hdeB* was amplified with primers: Tt1476; 5'-TCA TGTAACCGAAAAACGC CAATG and ThdeB; 5'-TTACC AGCGCTGCCACGATAAT-3'. PCR conditions were: 5 min initial denaturation at 94°C, 30 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 55°C and 1 min extension at 72°C, and a final extension step of 10 min at 72°C.

Primer extension analysis

Total RNA was isolated from *Salmonella* Typhi Ty2 grown aerobically using Trizol reagent (Life Technologies). To study *clyA* and *t1476* transcription, primer 5'-CGTCGCTCACTTA ACCGATAAGCATTTA-3', which was complementary to +66 to +94 of the transcription start site of *t1476*, was used. ^{32}P-labeled primer (50,000 cpm) was co-precipitated with 30 μg of total RNA. Primer extension reaction was performed as described previously (Shin *et al.*, 2001).

Construction of plasmids

To clone *clyA* operons, -196 to +209 fragment of *clyA* and *t1476* promoter, including *t1476* coding and their upstream sequences were amplified with primers: Pt1476-F; 5'-GCCGA ATTCGAACCTTATCAGGGCTAAACCAAC-3', and Pt1476-R; 5'-GCCGGATCCCGTGGTTGCAACAAAACTTAAACG-3'. The resulting PCR products were cloned into pCR 2.1-TOPO vector (Invitrogen) to generate plasmids pTPC2101 (Table 1). Plasmid DNA purified from one clone yielded an insert of the predicted size upon digestion with *Eco*RI and *Bam*HI, and the insert was ligated into a vector pRS415 (Amp^R) to generate plasmids pRPC4151 (Table 1).

β-Galactosidase assay

β-Galactosidase assay was performed as described previously (Miller, 1972) using cells permeated with Koch's lysis solution (Putnam and Koch, 1975). β-Galactosidase specific activity was expressed as Miller units ($OD_{420nm}\times1000/OD_{600nm}\times$

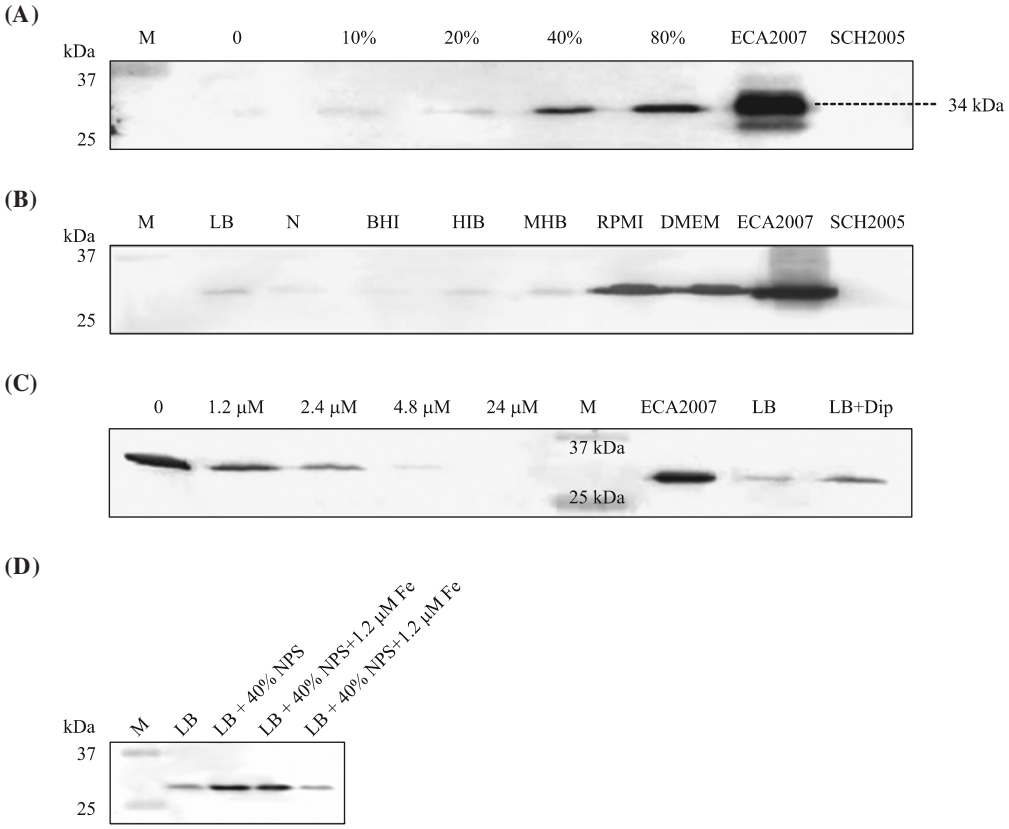

Fig. 1. Western blot analysis of ClyA expression in *S*. Typhi Ty2. *S*. Typhi Ty2 was cultured in LB broth overnight and subcultured in the test media for 4 h or 8 h. Bacteria were pelleted, resuspended in 1× PBS and diluted to A_{600}=1.0. One hundred microliters of boiling bacterial lysate was used for Western blotting. Strain ECA2007 overexpressing ClyA was included as a positive control and SCH2005 as negative control. (A) ClyA expression in *S*. Typhi Ty2 grown in LB supplemented with NPS at the indicated concentrations. *S*. Typhi Ty2 was subcultured in LB broth without or with NPS for 4 h. (B) ClyA expression in *S*. Typhi Ty2 in various media. *S*. Typhi Ty2 was subcultured in Luria-Bertoni (LB), Nutrient broth (N), Brain Heart Infusion (BHI), Heart Infusion broth (HIB), Mueller Hinton II broth (MHB), RPMI, and Dulbecco's Modified Eagle's Medium (DMEM) media for 8 h. (C) ClyA expression in *S*. Typhi Ty2 grown in RPMI supplemented with $Fe(NO_3)_3$ at the indicated concentration or LB broth supplemented with 0.1 mM 2,2'-dipyridyl. (D) ClyA expression in *S*. Typhi Ty2 grown in LB broth supplemented with 40% NPS or $Fe(NO_3)_3$. *S*. Typhi Ty2 was subcultured in LB broth, LB broth with 40% NPS, LB broth with 40% NPS and 1.2 μM $Fe(NO_3)_3$, and LB broth with 40% NPS and 12 μM $Fe(NO_3)_3$ for 8 h.

min×ml). Strains cultured overnight in LB broth were diluted to 1:100 in the indicated medium and were grown at 37°C until the culture reached stationary phase. Since RPMI was red-colored, the culture was washed with an equal volume of LB broth, and sampled for assay at regular time intervals. Each strain was assayed in triplicate and average enzyme activities were plotted as a function of time.

Results

Evaluation of ClyA expression in the presence of NPS and various media

To examine ClyA expression in *S*. Typhi Ty2, STJH1029 was cultured in LB broth supplemented with 10%, 20%, 40%, and 80% NPS, and subjected to Western blotting using rabbit anti-ClyA antiserum. The bacterial lysates were resolved by 12% SDS-PAGE gel and probed with rabbit anti-ClyA antiserum (Fig. 1A). ClyA migrating at 34 kDa was detected only when bacteria was cultured in the presence of a high concentration of NPS. ClyA expression increased in a NPS concentration-dependent manner, indicating that as-yet unidentified components of human serum promoted expression of ClyA in *S*. Typhi Ty2.

Since expression of ClyA in *S*. Typhi Ty2 was not significant in LB broth in the absence of NPS, we questioned whether other media could stimulate ClyA expression. Nutrient broth (N), Brain Heart Infusion (BHI) broth, Heart Infusion broth (HIB), Mueller Hinton II broth (MHB), RPMI, and Dulbecco's Modified Eagle's Medium (DMEM) were tested. Western blot analysis revealed significant expression of ClyA from the bacteria cultured in animal cell culture media, such as DMEM and RPMI (Fig. 1B).

Inhibitory effect of high-iron condition media on ClyA expression

LB broth and RPMI media were compared, and different components were analyzed to establish a correlation between ClyA expression and media condition. It was noted that there is no iron component in the animal cell culture media. Thus, bacteria grown in RPMI medium supplemented with increasing concentrations of $Fe(NO_3)_3$, and ClyA expression was ascertained by Western blot analysis (Fig. 1C). ClyA expression was inversely correlated with $Fe(NO_3)_3$ concentration. Similar observations were made with bacteria cultured in RPMI medium supplemented with $FeCl_3$ and $FeSO_4$ (data not shown). Conversely, we removed iron from LB broth by treating with the iron chelator 0.1 mM 2,2'-dipyridyl. Bacteria grown in this medium expressed increasing levels of ClyA (Fig. 1C). We also added 1.2 and 12 μM $Fe(NO_3)_3$ to LB broth supplemented with 40% NPS; ClyA expression was reduced concomitantly with increasing iron concentration (Fig. 1D). Taken together, these findings are consistent with the suggestion that iron can inhibit expression of ClyA in *S*. Typhi Ty2.

Regulation of *clyA* transcription by iron

clyA-like open reading frame of serovar Typhi S2369/96 and CT18 displays 100% nucleotide sequence identity with the *clyA* coding sequence of the serovar Typhi vaccine strain Ty21a and 87% nucleotide sequence identity with *E. coli* *hlyE*, but the upstream region of *S*. Typhi *clyA* is different from that in *E. coli* K-12 (Oscarsson *et al*., 2002). Presently, we also compared the region near *clyA* between *S*. Typhi Ty2 and *E. coli* K-12, and found a dissimilar gene arrangement (Fig. 2A). To identify the promoter region of *clyA*, RT-PCR was performed with total RNA using specific primers. RT-PCR using the primers amplifying the region between *clyA* and *t1476* generated a product, while amplifying the region between *t1476* and *hdeB* did not (Fig. 2A and B). This result was consistent with the suggestion that the *t1476* and *clyA* genes are transcribed together as a polycistronic mRNA. Hence, we speculated that the promoter for *clyA* (abbreviated as *clyA*p) should be located upstream of *t1476*. Primer extension was used to determine the start site of transcription in *t1476* using total RNA. Transcription was observed to start from the "C" residue 140 bp upstream from the translational start site, TTG (Fig. 2C and D). A putative -10 (TATAAC) and -35 (AGTAGA) region was deduced from the DNA sequence. To examine its regulation, the *t1476* promoter region (-196∼+209) was cloned into the *lacZYA* transcription vector pRS415 and its activity was measured by a β-galactosidase assay. Bacteria were grown in RPMI medium (which is low in iron) or RPMI medium containing 24 μM $Fe(NO_3)_3$, and β-galactosidase activities were determined during the course of growth. β-Galactosidase activity under both low and high iron conditions increased 2-fold as the culture entered stationary phase. The activity was approximately 2-fold higher under the low-iron condition in comparison with the high-iron condition. Thus, the *t1476* promoter is more or less stationary phase inducible and is subject to iron regulation, but only marginally, suggesting that iron regulation of ClyA expression would be at translational level for the most part.

Discussion

Iron is considered to be a limiting factor in the growth of various pathogenic bacteria (Sritharan, 2006). Free iron is extremely limited in the mammalian host, and its concentration was approximately 10^{-18} M in normal human serum (Bullen *et al*., 1978), while the concentration of iron in yeast extract used in making LB broth is about 5×10^{-3} M. LB broth and NPS completely lacking free iron were chosen to compare their effects on ClyA expression in *Salmonella* Typhi. Expression of ClyA was detected significantly from *S*. Typhi Ty2 grown in LB only in the presence of NPS in its concentration dependent manner (Fig. 1A). The ClyA was detected in *S*. Typhi grown in iron absent media DMEM or RPMI (Fig. 1B), and was significantly decreased when RPMI was supplemented with iron (Fig. 1C). Thus, ClyA is expressed in iron-depleted condition. Most intracellular iron is found as hemoglobin, heme, ferritin, and hemosiderin, and extracellular iron is bound by the high-affinity iron-binding glycoproteins transferrin and lactoferrin. Unsaturated transferrin appears to contribute to the bacteriocidal activity of normal human plasma, while lactoferrin may serve a similar function on secretory surfaces, and is also an important component of phagocytic cells. Extracellular hemoglobin and heme have been found to serve as iron sources for many bacteria; however, they are rapidly bound by two serum

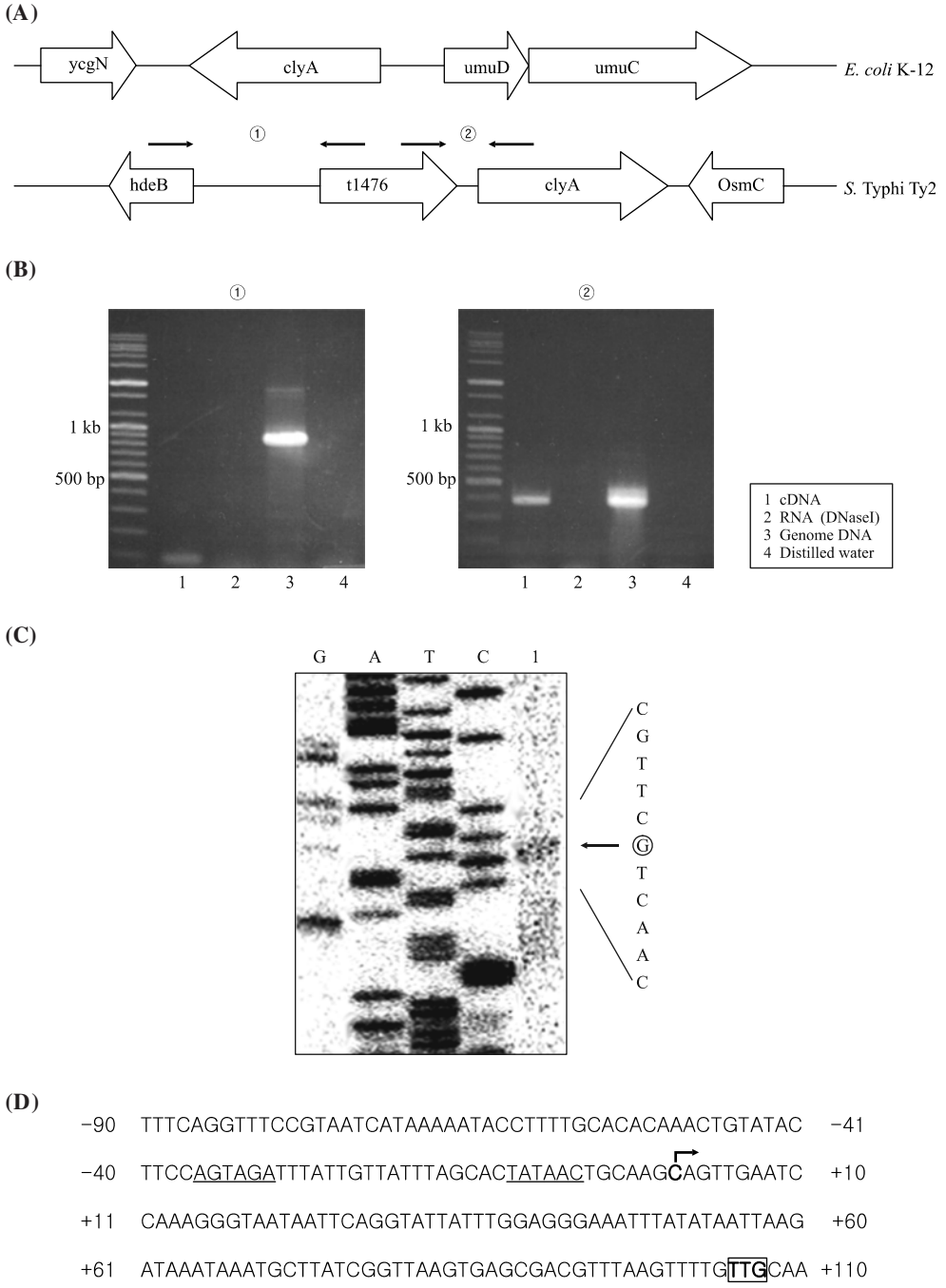

Fig. 2. Analyses of *clyA*. (A) Schematic map of the gene arrangement containing the *clyA* locus in *E. coli* K-12 and *S*. Typhi Ty2. (B) RT-PCR products amplifying the region between *hdeB* and *t1476* ①, and *t1476* and *clyA* ②. (C) Primer extension analysis of RNA isolated from STJH1029. Lanes G, A, T, and C are the sequence ladders for the promoter region of *t1476*; lane 1 shows the primer extension products from the upstream of *t1476*. (D) Nucleotide sequence of the promoter region of *t1476*. The transcription start site of *t1476* and *clyA* is shown with arrows, the -10 and -35 regions of the promoter are underlined, and the start codon TTG is indicated with box.

proteins, haptoglobin and hemopexin, respectively (Litwin and Calderwood, 1993). To survive and multiply in the host, bacteria have to express virulence determinants or toxins to acquire free iron. Therefore, iron can be considered as a critical determinant of virulence expression (Payne and Finkelstein, 1975). However, the existence of iron-regulation in *Salmonella* Typhi has never been reported to the best of our knowledge.

Bacteria utilized a number of mechanisms to take up iron from the host. Many bacteria produce high-affinity iron chelators called siderophores, which compete effectively with the host iron-binding compounds transferrin and lactoferrin in response to iron stress (Neilands, 1981, 1982). Another mechanism for iron acquisition by bacteria is the produc-

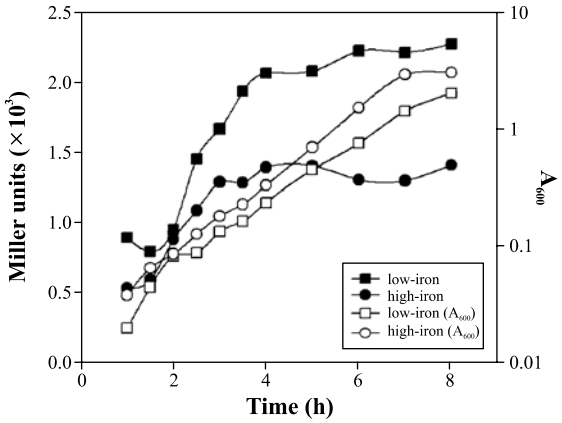

Fig. 3. Effect of iron limitation on *clyA* expression during the growth of *S*. Typhi Ty2. STJH1106 strains that carry pRPC4151 (*clyAp*::*lacZYA*) were cultured in RPMI media (low-iron condition) and RPMI media containing 24 μM $Fe(NO_3)_3$ (high-iron condition). The *clyA* promoter activities were measured by a β-galactosidase assay. Growth curves of STJH1106 in low-iron condition (square) and high-iron condition (circle) are shown and the OD_{600nm} values are indicated on the right. Similar results were obtained from two additional and independent experiments.

tion of hemolysin (Stoebner and Payne, 1988; Ochsner *et al*., 2000). Hemolysins appear to promote infection by providing iron to the pathogen *in vivo* (Linggood and Ingram, 1982; Waalwijk *et al*., 1983); interestingly, it has been found that synthesis of such a hemolysin is always controlled by iron availability in *E. coli* (Waalwijk *et al*., 1983), *Streptococcus pyogenes* (Griffiths and McClain, 1988), *Vibrio cholerae* (Stoebner and Payne, 1988), and *Serratia marcescens* (Poole and Braun, 1988). Other bacterial pathogens use heme as a source of iron, including *Shigella dysenteriae*, *Yersinia enterocilitica*, *Yersinia pestis*, *Neisseria* spp., *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Haemophilus influenzae* (Neilands, 1981). Those genes required to acquire iron are normally silent during iron-sufficient growth (Crosa, 1997; Braun, 1998). Since ClyA of *S*. Typhi Ty2 causes hemolysis and its expression increases under low-iron condition (Fig. 1C and D), we speculate that *S*. Typhi Ty2 also utilizes hemolysin by expressing ClyA to acquire iron in their host.

Iron is as a regulatory signal. Other virulence determinants controlled by iron concentration include Shiga toxin from *Shigella dysenteriae* type I (Strockbine *et al*., 1988), Shiga-like toxin I (SLT-I) from enterohemorrhagic *E. coli* (Calderwood and Mekalanos, 1987), diphtheria toxin from *Corynebacterium diphtheriae* (Homma *et al*., 1963; Murphy *et al*., 1978), exotoxin A from *Pseudomonas aeruginosa* (Bjorn *et al*., 1978), and iron-regulated outer memberane proteins from *Yersinia* spp. (Carniel *et al*., 1987). Because ClyA possesses cytotoxic activities, the protein may be regulated by iron as a sort of toxin.

Regulation of bacterial gene expression is mainly at the transcriptional level. To determine whether the transcription of *clyA* was affected by iron, the *clyAp*::*lacZYA* transcription fusion was constructed, and β-galactosidase activity was determined in *S*. Typhi grown under high- or low-iron conditions. The results showed that *clyA* promoter activity in low-iron condition was only about 2-fold higher than in the high-iron condition (Fig. 3), which was much less than that anticipated based on determination of ClyA by Western analysis (Fig. 1). Thus, it is likely that the iron-mediated regulation of ClyA is at a step subsequent to transcription initiation, translation, or protein turn-over.

Iron controls diverse virulence determinants in bacteria. Here, we demonstrate that the expression of ClyA protein in *S*. Typhi Ty2 is negatively regulated by iron, suggesting that free iron depletion is one of the triggers inducing ClyA production in *S*. Typhi during host infection.

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