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Initial Characterization of *yliH* in *Salmonella typhimurium*

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Using microarray analysis, we determined those *Salmonella* genes induced at the entry of stationary phase, and subsequently discovered that uncharacterized *yliH* was induced most dramatically. We set out to establish the molecular mechanism underlying the stationary phase induction of *yliH* under the standard culture condition, LB with vigorous aeration, by analyzing its promoter activity in various mutant backgrounds, lacking stationary phase σ, RpoS, or stringent signal molecules ppGpp, Δ*relA* Δ*spoT*. It was found that the stationary phase induction of *yliH*p was partially dependent on *rpoS* but entirely dependent on ppGpp. DNA sequence analysis revealed that the *Salmonella yliH* gene is composed of 381 base-pair nucleotides, with overall amino acid sequence revealing 76.38% amino acid identity and 88.98% similarity with *Escherichia coli yliH*, although no motif from data base was noted for its possible role. Recently however, it has been reported that *yliH* in *E. coli* was implicated in biofilm formation and motility by repressing these activities (Domka *et al.*, 2006). We have constructed a mutant *Salmonella* deleting *yliH* gene by allele replacement and examined its phenotype, and found that the *yliH* in *Salmonella* more or less affects motility and adherence by enhancing these activities. The effect on biofilm formation in *Salmonella* was uncertain. Moreover, addition of cloned *yliH* of *E. coli* into *Salmonella* did not reduce motility or adherence. Taken together, it appears that the pathways implicating *yliH* for biofilm formation and motility in *E. coli* and in *Salmonella* are somewhat different.

Keywords: *Salmonella typhimuriums*, *yliH*, biofilm, ppGpp, *rpoS*

Enteric *Salmonella* infection is a global problem both in human and animals, and is regarded to be the most important bacterial etiology for enteric infections worldwide (Fierer and Swancutt, 2000). The serotype *Salmonella enterica* serovar Typhimurium remains the most frequently isolated in human, swine, avian, and bovine salmonellosis (Popoff and Le Minor, 1997), and can cause a systemic, typhoid-like disease in mice (Coburn *et al.*, 2005). The ability of enteric bacteria to survive and multiply in the animal gastrointestinal environment is important for establishing infection and maintaining survival. This survival strategy requires the ability to sense changes in environmental conditions and to respond with the appropriate gene regulation.

Transcription in bacteria is catalyzed by RNA polymerase (RNAP), a multisubunit enzyme composed of the core enzyme (subunit composition, $\alpha_2\beta\beta'$) with the catalytic activity of RNA polymerization and the sigma subunit (σ) recognizing promoter elements on the DNA. Bacteria have one housekeeping sigma factor (σ^D) and a variable number of alternative σ factors that possess different promoter-recognition properties. Alternative σ factor control specialized regulons that are activated during specific stress conditions and growth transitions. In *Escherichia coli* and *Salmonella* spp., σ^S, encoded by *rpoS*, the master regulator of the general stress response is essential for transcription of some stationary phase-specific genes (Hengge-Aronis, 2002). The synthesis and accumulation of σ^S are controlled at multiple levels (Loewen and Hengge-Aronis, 1994). Transcription control of *rpoS* has been shown to involve a number of factors, including ppGpp and polyphosphate as positive regulators and cyclic AMP and UDP-glucose as negative regulators among many others (Loewen and Hengge-Aronis, 1994). ppGpp is an effector molecule of the stringent control modulation, synthesized by RelA and SpoT (Cashel *et al.*, 1996). Entry into the stationary phase as well as growth arrest associated with nutrient shift presumably induces accumulation of ppGpp (Cashel *et al.*, 1996). As concentration of ppGpp increases, transcription of stable RNA genes is ceased while other's encoding function to cope with the stress condition is enhanced (Gentry *et al.*, 1993).

A biofilm is a functional consortium of microorganisms organized within an extensive exopolymer matrix comprised mainly of hydrated polysaccharides (Whitfield and Keenleyside, 1995). It has been suggested that the production of biofilm may enhance the survival of cells in dynamic environments by allowing the formation of colonies containing thousands of cells. Various stresses invoke this polymer formation as part of a general stress response from microorganisms (Fux *et al.*, 2005). Thus, stationary-phase cultures also produce a thin layer of biofilm on the walls of culture tubes (Christensen *et al.*, 1982). Biofilms are produced by a wide variety of environmentally and medically important microorganisms, including

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Staphylococcus, *Pseudomonas*, *Desulfovibrio*, *Haloferax*, *Thermococcus*, *Methanosarcina*, and *Methanobacterium* (Watnick and Kolter, 2000). It has been suggested that Typhoidal *salmonellae* may survive within their asymptomatic human hosts by forming biofilms on the surfaces of gallstones (Prouty *et al.*, 2002). In the case of pathogenic *E. coli* strains, biofilm formation has been considered essential in the colonization of the human gastrointestinal tract, although numerous factors have been implicated in the process (Torres *et al.*, 2005). Recently, two genes, *yliH*, a putative receptor that is induced in the stationary phase, and *yceP*, a conserved hypothetical protein, have been implicated in *E. coli* biofilms (Ren *et al.*, 2004). Deletion of *yceP* or *yliH* has been shown to increase biofilm formation in continuous-flow chambers with minimal glucose medium (Domka *et al.*, 2006). Biofilm formation has been linked to expression of motility related genes (Pratt and Kolter, 1998). Consistently, it was reported that the mutation in the either one of above genes increased bacterial motility.

In this study, we report the cloning and initial characterization of *yliH* gene in *Salmonella typhimurium*. The *yliH* gene was found to be one the most significantly induced as *Salmonella* culture entered stationary phase as assessed by microarray analysis. It was found to be structurally similar to that in *E. coli*, expressed at the onset of stationary phase in ppGpp-dependent manner. Mutation in the *yliH* gene was found to decrease motility and adhesion while not affecting biofilm formation.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains, which were derived from WT *S. typhimurium* 14028s, and the plasmids used in this study are listed in Table 1. All bacterial strains were constructed by P22HT *int* transduction as described previously (Davis *et al.*, 1980). The *yliH::kan* strain was constructed following the method developed by Datsenko and Wanner (2000). Briefly, PCR primers were designed to replace *yliH* gene to *kan* marker gene as described; 5' primer-AGAACCGATCTTCTCAACAAGCTGATAAACGCGCGTATCGGTGTAGGCTGGAGCTGCTTC and 3' primer-TTGTTCAGACCTGTGTAAGCGGTGAATGTGCTTTCAGACTATTCCGGGGATCCGTCGACC, which consisted of 40 nt *yliH* (*yliH* sequences are underlined) and 20 nt *kan* marker gene sequences. PCR were conducted with these primers set against pKD13 carrying *kan* marker gene.

The 1.4 kb PCR products were purified and transformed into bacteria carrying a Red helper plasmid (pKD46) by electroporation.

The electrocompetent cells were then grown in Luria-Bertani (LB) broth with ampicillin and L-arabinose (1 mM) at 30°C to an A_{600} of $\approx$ 0.5. The mutants were confirmed by PCR using kan primers: k1; 5'-CAGTCATAGCCGAATAGCCT and kt; 5'-CGGCCACAGTCGATGAATCC, and *yliH* primers: 5' primer; 5'-GAACCCTGGTAGAAAAGAGG and 3' primer; 5'-TGTCTTGGTTTGCTGGGGAT.

Growth conditions

Except indicated otherwise, bacteria were grown in LB medium (Difco Laboratories) containing 1% NaCl with vigorous aeration at 37°C. For solid support medium, 1.5% granulated agar (Difco Laboratories) was included. Minimal salts medium of Vogel-Bormer (VB) (Kano *et al.*, 1968), supplemented with 0.02% or 0.4% glucose was used. The concentration of antibiotics (Sigma, USA) used in this study were

Table 1. Strains and Plasmids

Strains	Description	Reference or source
S. typhimurium		
14028s	Wild type	
SMR2160	*yliH::kan*, Kan^r	This work
SAL2006	*putA::T1*$_4$:: -207~+87 of *yliH::lacZ::cat*, Cam^r	This work
SAL2007	*rpoS::Amp*, *putA::T1*$_4$::-207~+87 of *yliH::lacZ::cat*, Amp^r, Cam^r	This work
SAL2008	*relA::kan*, *spoT::cat*, *putA::T1*$_4$::-207~+87 of *yliH::lacZ::cat*, Cam^r, Kan^r	This work
KH004	*yliH::kan*, pBAD18, Kan^r, Amp^r	This work
KH005	*yliH::kan*, pKH001, Kan^r, Amp^r	This work
KH006	*yliH::kan*, pKH002, Kan^r, Amp^r	This work
E. coli		
K-12 MG1655	Wild type	
Plasmid		
pRS415	*LacZ* fusion vector, Amp^r	Simons *et al.*, 1987
pHN002	pRS415, *ΔlacYA*, *Cat* fused to *LacZ*, Cam^r	This work
pMR020	pHN002 containing -207 to +87 of *yliH*	This work
pBAD18	Amp^r	
pKH001	*yliH* ORF of *S. typhimurium* cloned into pBAD18, Amp^r	This work
pKH002	*yliH* ORF of *E. coli* cloned into pBAD18, Amp^r	This work

100 μg/ml ampicillin, 15 μg/ml chlorampenicol, and 50 μg/ml kanamycin. X-gal (Sigma, USA) was used at 20 μg/ml.

Introduction of an in-frame yliH-lacZ fusion gene into the S. typhimurium chromosome

S. typhimurium strains carrying *lacZ* genes transcriptionally fused to *yliH* promter on the chromosome were constructed by the modification of the method developed by Datsenko and Wanner (2000). Briefly, we cloned *yliH* promoter region (-207~+87) into pHN002 which carryied *lacZ* structural gene and *cat* gene in the place of *lacYA* in pRS415, a vector system for the *in vitro* construction of transcriptional fusions to the *lacZ* gene (manuscript in preparation). This plasmid (pMR020) was digested with *Pst*1 and purified. The *yliH*p::*lacZ*::*cat* was amplified with primers that contain sequences for *putA* site of *S. typhimurium*; 5' primer-GAAATCGCCTG TTAATGGTACCAATAGCCTTGACGCAATAGAGTAATG ACCGAGGCCCTTTCGTCTTCAAGAATT and 3' primer-CGTCATTGTCAGTCTCTTACAGAAAGATTACACGATT ATTTCATCGGCAGGAGACGTGTGTAGGCTGGAGCTG CTTC, which consisted of 50 or 55 nt *putA* (*putA* sequences are underlined) and 25 or 22 nt sequences of pHN002. PCR were conducted with these primer set against *Pst1* digested pMR020.

The 5.3 kb PCR products were purified and transformed into bacteria carrying a Red helper plasmid (pKD46) by electroporation. This *putA*::*yliH*p::*lacZ*::*cat* construct was confirmed by PCR.

β-Galactosidase assays

β-Galactosidase assays were performed as described by Miller (1972) using cells permeabilized with Koch's lysis solution (Putnam and Koch, 1975). β-Galactosidase- specific activity was expressed as A_{420}/min/A_{600}/ml units. To measure β-galactosidase activities in bacteria at different stages of growth, overnight culture of bacteria was diluted 1:50 into LB, as previously described and grown at 37°C until the growth reached the stationary phase. Samples were taken for enzyme assay at regular time intervals. The β-galactosidase activities for each bacterial strain were assayed in triplicate and average enzyme activities plotted as a function of time.

Motility assay

Bacteria cultured overnight in LB broth were used for motility assay performed at 37°C on 0.3% LB agar plates. The motility diameters were measured at 8 and 16 h. Eight plates were used to evaluate motility for each independent culture.

Bacterial adherence in intestine

Bacterial adherence in intestine were assayed according to Alam *et al.* (1996). The small intestine of young chicken was excised and opened by longitudinal incision. They were then washed with sterile normal saline and fixed with 10% formaline in Krebs-Ringer-Tris (KRT) buffer for 16 h. Subsequently, the formaline-fixed intestine was washed with the buffer and was punched into 10-mm-diameter circles.

Each strain of bacteria cultured overnight in LB broth was sub-cultured for 4 h at 37°C. The bacterial cells, harvested by centrifugation at 4,000 rpm for 5 min, were suspended in KRT buffer, and number of bacteria in the suspension was adjusted to 5×10^9 CFU/ml. The intestinal piece was dipped into the suspension and subsequently incubated at room temperature for 15 min with mild agitation. The intestinal piece was washed with KRT buffer to remove non-adherent bacteria, and placed in a glass homogenizer containing 1 ml of the buffer. The homogenate was diluted serially and plated on LB agar. After 1 day of incubation at 37°C, discrete colonies were counted. The adherence index was defined as the average number of bacteria per 10-mm-diameter piece of intestinal mucosa.

Microtiter biofilm assay and tube method

Microtiter biofilm assays were conducted using the protocol described by O'Toole *et al.* (1999). Two types of media were employed; LB broth and LB broth supplemented with 0.4% glucose (LB-glu). Sterile 96-well flat-bottomed polystylene microplate was filled with 180 μl of the appropriated medium. The negative control wells contained media only. A quantity of 20 μl of overnight bacterial culture was added into each well. Microtiter plates were then sealed with parafilm, and incubated with gentle shaking at 37°C or 28°C for 48 h. The assay was tested four times against each strain. To measure biofilm formation, the content of the plate was poured off and the wells were washed 2 times with 200 μl of sterile distilled water. Two hundreds microliter of 0.1% (w/v) crystal violet was added into each well and incubated at room temperature for 20 min. The crystal violet and unattached cells were aspirated using a pipette. Each well was then rinsed gently with two washes of 200 μl distilled water, allowed to dry at room temperature for 15 min, and observed. A qualitative assessment of biofilm formation was determined as previously described by Christensen *et al.* (1982). LB was inoculated with loopful of microorganism from overnight culture plates and incubated for 48 h at 37°C or 28°C. The tubes were decanted and washed with sterile distilled water and dried. Dried tubes were stained with crystal violet (0.1%). Tubes were then dried in inverted position and observed for biofilm formation.

Results and Discussion

Cloning and investigation of the S. typhimurium yliH gene

We compared the transcripts obtained from the *S. typhimurium* 14028s at exponential ($A_{600}\leq1$) and early stationary phase ($A_{600}\leq3$) under standard culture conditions to determine the expression profile of the genes induced at each growth phase. Since the stationary phase σ, RpoS, and the stringent signal molecule, ppGpp, have been implicated in the stationary phase gene induction, we also examined the transcripts in the mutant defective within the respective mutant genes of RpoS$^-$, and Δ*relA*Δ*spoT*. Comparative gene analysis was performed using microarray containing the 4622 predicted open reading frames (ORF) of *S. typhimurium* (McClelland *et al.*, 2001). It was revealed that the expression of ~250 ORF were altered as bacterial culture entered stationary phase (data not shown). Among the induced genes at the stationary phase, *yliH* was remarkable, of which expression was increased up to 25 fold compared with that at the exponential phase in wild type *Salmonella*. *yliH* expression was fur-

ther increased to 66.5-fold in RpoS- mutant background, but reduced more than 20 fold in ppGpp-defective mutant (Table 2).

Subsequently, in an attempt to further study the *yliH* in *Salmonella*, we cloned the gene and analyzed its DNA sequence. The sequence analysis revealed 381 bp ORF (Fig. 1). A putative ribosomal binding sequence (RBS) was found 10 bp upstream from translational start site (ATG). Putative -10 hexamer, TAAAAA, and -35 hexamer, TTGATC, separated by 17 bp spacer were identified at 35 bp upstream of the RBS. A at 7 bp downstream of the putative-10 sequence was assigned to be putative transcription start site (+1). The *yliH* gene in *S. typhimurium* was 74% identical in DNA sequence to that in *E. coli* (Fig. 2). The *S. typhimurium yliH* gene encodes 127 amino acid protein, which is similar in size to reported YliH proteins in *E. coli*. It is homologous to the *E. coli* YliH protein with 76.38% amino acid identity and 88.98% similarity. However, we have not found any motif from data base that indicates possible role of YliH.

Induction of yliH at stationary phase

To verify the expression pattern of *yliH*, its promoter activity was determined using *lacZ*-fusion construct. The DNA segment containing *yliH* promoter fused to *lacZ* was placed at near *putA* site on chromosome using a modified linear DNA transformation system of Datsenko and Wanner (2000) (manuscript in preparation). This system allowed monitoring promoter activity during the bacterial growth into stationary phase without possible complication of changing copy number of the promoter during culture. Bacteria were grown under standard condition, LB with vigorous aeration, and samples extracted at regular time intervals for β-galactosidase assay. The β-galactosidase activity was found to remain at the basal level during the exponential phase of growth, but increased over 50 fold concomitantly with growth transition into stationary phase (Fig. 3, closed circles). This result was consistent with the findings of cDNA microarry analysis (Table 2).

We then set out to establish the molecular mechanism underlying the stationary phase induction of *yliH* under the standard growth condition. The β-galactosidase activity was determined using the same *yli*Hp::*lacZ* chromosome fusion in RpoS$^-$, or ppGpp-defective mutant (*ΔrelAΔspoT*) background (Fig. 3). Growth of the RpoS$^-$ or ppGpp-defective mutant were not overly different from the wild type strain under the standard growth condition (data not shown). In the RpoS$^-$ mutant, *yliH*p was found to be only partially induced at the entry into stationary phase (Fig. 3, closed triangles). However, *yliH*p activity was completely silent throughout the growth phase in the mutant strain lacking ppGpp (Fig. 3, closed squares). Thus, it seems that the stationary phase induction of *yliH*p was not entirely independent of *rpoS* but dependent entirely on ppGpp. This result suggested a possibility that there may be two promoters driving the expression of yliH gene, one RpoS dependent and another independent promoter. In general, ppGpp-defective mutant and the RpoS- mutant have similar phenotype: this has been explained by

Table 2. Expression pattern of *yliH* gene expression during *Salmonella* growth into stationary phase under the standard conditions.

Gene	Fold activation in WT	Fold activation in RpoS-	Fold activation in *ΔrelAΔspoT*
yliH	25.52	66.50	0.09

Fold differences between growth phases in each strains were estimated from the differences of M values, which were calculated as log2 (sample/reference)

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gttccgaatcaacgaggtttttcggacagcccagggaaacgaagccgatct
ttggctggtgggttacattactcatagcttaaaaagtgttcatttattgga
atggtcagggcagggattctacagatatccgaacaaaaattgtatcgcaat
ctcatcaggagcggcggcatcaaccgttgtggttatcagcaaaaggtgaaa
aagggcaattctaagaaaatttcttgatctctgacacccctttgtctaaaaa
acgaccatgctattaagttgaaccctggtagaaaagaggaggaaaaaaggt
atggtcgttgacagactgagaaccgatcttctcaacaagctgataaacgcg
 M  V  V  D  R  L  R  T  D  L  L  N  K  L  I  N  A
cgtatcgatcttgccgcttatctgcaattgcgaaaagcgaaggggtatatg
 R  I  D  L  A  A  Y  L  Q  L  R  K  A  K  G  Y  M
tcagtcagcgaaagtgacactctgcgtgacaatttttttgaactgaatcgc
 S  V  S  E  S  D  T  L  R  D  N  F  F  E  L  N  R
gagctccacgatcacgctttacggcagggactgcatctcgatcaggaagag
 E  L  H  D  H  A  L  R  Q  G  L  H  L  D  Q  E  E
tggaacgcgctgcgccgtgccgaaggcgcgcttgcagccgccgccgtttgt
 W  N  A  L  R  R  A  E  G  A  L  A  A  A  A  V  C
ttaatgagcggacaccatgattgtccgacctttattgccgtcaacgcagat
 L  M  S  G  H  H  D  C  P  T  F  I  A  V  N  A  D
aaacttgaaaactgtctgacaactttgacgctgagtattcagagtctgaaa
 K  L  E  N  C  L  T  T  L  T  L  S  I  Q  S  L  K
gcacattcaccgcttacacaggtctga
 A  H  S  P  L  T  Q  V  *
```

Fig. 1. DNA sequence analysis of *S. typhimurium yliH* genes. Start codon and stop codon are written in bold. RBS (ribosome binding site) is underlined. Deduced amino acid sequences are written as a one-letter code. Putative -10 and -35 sequences are boxed. Putative transcription start site (+1) is circled.

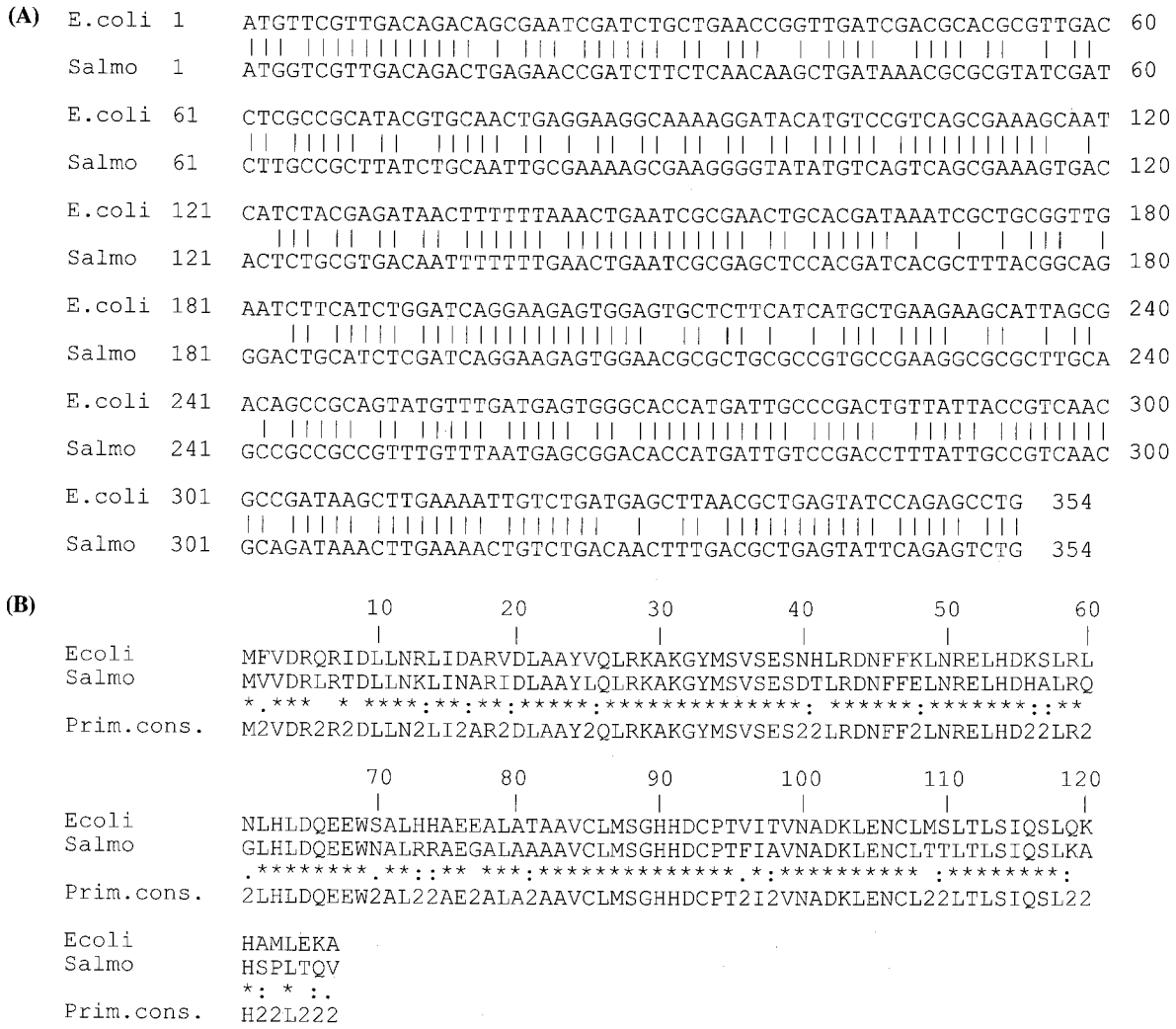

Fig. 2. Alignment of the DNA sequence (A) and deduced amino acid sequence (B) of the *yliH* in *E. coli* (upper row) and *S. typhimurium* (lower row). * indicates identical amino acid.

the fact that ppGpp-deficient mutants fail to induce the *rpoS* (Gentry *et al.*, 1993). The transition into stationary phase represents a stress environment that is sensed and translated into ppGpp accumulation in bacteria (Cashel *et al.*, 1996; Song *et al.*, 2004). In ppGpp-defective mutant, *rpoS* expression remains at the basal level. In addition, ppGpp is required for functional activity of the stationary phase RNA polymerase carrying σ^S, RpoS, presumably to load σ^S onto the core in place of vegetative σ^D (Kvint *et al.*, 2000; Jishage *et al.*, 2002). It is, therefore, perceived that ppGpp is the signal molecule at the entry into the stationary phase that allows RpoS-dependent transcription and also directly activates those RpoS-independent stationary phase genes. In this sense, stationary phase gene induction could be considered as the ppGpp mediated gene activation, although exact mechanism has yet to be defined (Xiao *et al.*, 1991; Choy, 2000; Barker *et al.*, 2001; Paul *et al.*, 2005; Magnusson *et al.*, 2007). This class of genes includes *hilA* gene in *S. typhimurium*, a master regulator of *Salmonella Pathogenecity Island I genes*, that is expressed at the entry of stationary phase. The induction is independent of *rpoS* but dependent entirely of ppGpp (Song *et al.*, 2004). It might be possible that the elevation of ppGpp level at the entry of stationary phase inhibits stable RNA synthesis, comprising bulk of transcription during exponential phase of growth that results in the transcription initiation from those stationary phase genes (Cashel *et al.*, 1996). Similar mechanism has been suggested for ppGpp-dependent transcription activation (Zhou and Jin, 1998; Barker *et al.*, 2001). This model predicts that the limiting rate for these ppGpp-dependent stationary promoters would be RNAP binding step. Alternatively, these genes might be activated directly by ppGpp (Choy, 2000; Paul, 2005).

Phenotype associated with YliH mutant

To examine the function of *yliH*, we disrupted the gene by an allele replacement method (Datsenko and Wanner, 2000). The Δ*yliH* strain was grown in LB or VB minimal media [no difference was observed between wild type and YliH⁻ mutant (data not shown)]. Since we have not found any

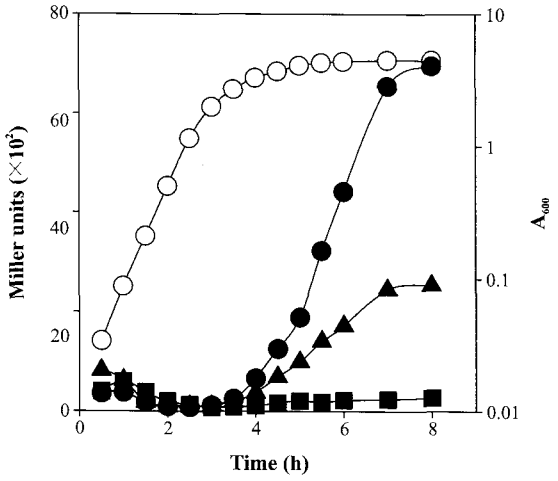

Fig. 3. *yliH*p activity in WT, RpoS⁻, and ppGpp defective mutant backgrounds during the growth under standard condition. The curve with open symbols represents growth (A_{600}), and the curve with closed symbols represents the activity of each promoter as determined by the β-galactosidase assay. Circles are for the WT strain, and triangles for RpoS-, squares for ppGpp-defective mutant.

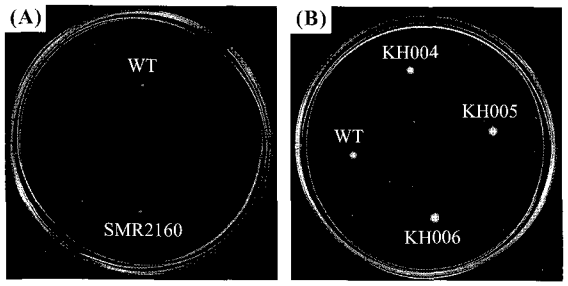

Fig. 4. Motility assay of WT or YliH⁻mutant (*yliH::kan*). Overnight broth-cultured cell was dropped on the 0.3% LB agar plate and incubated for 8 h. (A) shows WT and YliH⁻ mutant. (B) shows WT, KH004 (YliH⁻mutant, pBAD18), KH005 (YliH⁻mutant, pBAD18 *yliH*⁺ ORF of *S. typhimurium*), and KH006 (YliH⁻mutant, pBAD18 *yliH*⁺ ORF of *E. coli*).

motif from data base that indicates possible role of YliH, we examined the mutant phenotype based on the reported *E. coli yliH* function. In *E. coli*, YliH⁻ mutant has been reported to be hyper-motile in accordance with increased transcription of the flagella and motility loci, and enhanced biofilm former, without mechanistic details (Domka *et al.*, 2006). To study the implication of *yliH* in motility, we compared swarming phenotype of wild type and YliH⁻ mutant on LB swarm plate (0.3%) (Fig. 4). The chemotactic ring of *Salmonella yliH*⁻ was found to be smaller than that of WT (Average of 8 times trial). It therefore seems that YliH have an effect on the motility of *Salmonella*, although opposite to that in *E. coli*.

Formation of biofilm should be preceded by bacterial adherence to a surface for multiplication and production of extracellular polymers (Marshall *et al.*, 1971, 1992). Since *yliH* in *E. coli* was found to affect biofilm formation, we first examined the involvement of *yliH* gene during colonization of *S. typhimurium* by testing ability of the mutant to adhere to the intestinal mucosa. The mutant and wild type *Salmonellae* were tested for adherence using formalin-fixed chicken intestine. It was observed that YliH⁻ mutant adhered slightly less than that of the wild type (at least 10^7 cells attached to 1 cm^3 intestinal piece by wild type, but the 10^6 or fewer cells by YliH⁻ mutant). Therefore, it appeared that adhesion and motility were reduced by the mutation in the *yliH* gene in *Salmonella*. To further verify that the mutation in *yliH* gene was responsible for the altered motility and adhesion phenotype, it was complemented in *trans* using the plasmid (pBAD) carrying *yliH* of *E. coli* as well as of *Salmonella*. The complementation with *Salmonella yliH*⁺ (in the presence of 0.2% arabinose) led to increase in motility (Fig. 4B) and adhesion ($\sim 10^7$ cells/1 cm^3) back to the level of the wild type *Salmonella*. Most interestingly, the complementation with the multicopy *E. coli yliH*⁺ resulted in the same increase in motility (Fig. 4B) and in adhesion ($\sim 10^7$ cells/1 cm^3) as with the *Salmonella yliH*⁺. Evidently, the phenotype associated with *Salmonella yliH* mutation was inconsistent with that reported in *E. coli*.

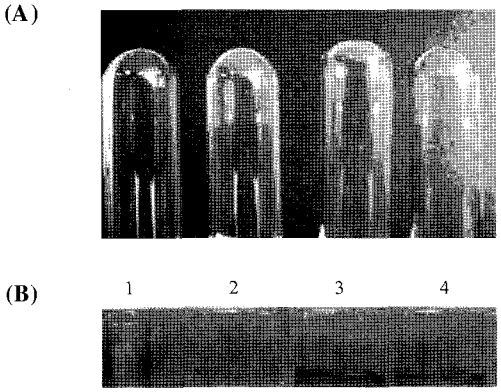

Fig. 5. Biofilm formation. Detectable biofilm in LB supplemented with 0.4% glucose (3.4) was shown by crystal violet staining. The images were obtained 48 h after bacterial incubation in the tube or well at 28°C. (A) Tube method, (B) Microtiter plate method. 1, WT in LB media; 2, YliH⁻ mutant in LB media; 3, WT in LB-glu media; 4, YliH⁻ mutant in LB-glu media.

Lastly, we examined the role of YliH in biofilm. The biofilm-forming abilities of the YliH⁻ mutant was assessed in LB and LB-glu media or a starvation medium (VB minimal) at 37°C. Biofilm formation was quantified after 24 h to 48 h incubation in LB or VB minimal media in 96-well polystyrene tissue culture plate and tube method (Christensen *et al.*, 1982; O'Toole *et al.*, 1999). The biofilm is quantified by staining with crystal violet as described our methodology. Neither WT nor YliH⁻ mutant formed a detectable biofilm in each media at 37°C. Lack of biofilm formation at 37°C by laboratory strains of *Salmonellae* 14028s has been previously reported (Romling *et al.*, 1998). We examined biofilm formation at 28°C in LB and in LB-glu media. In overnight liquid cultures at 28°C, we detected that biofilm formed at the wall of the glass and polystyrene tube by WT *Salmonella* in LB-glu media condition (Fig. 5). Under the same condition, we detected similar level of biofilm for-

mation by the YliH⁻ mutant. The *Salmonella* carrying extra copy of yliH (pBAD *yliH*+) of *E. coli* or *Salmonella* was also tested and found that neither produced discernable quantity of biomass .

We noted that the crystal violet staining technique is only a measure of gross biofilm formation. Thus, it is possible that mutants that appeared to be unaffected by this assay could have had a different structure of biofilm that was not detected by the crystal violet staining technique. We also constructed fluorescent-tagged *Salmonellae* and analyzed biofilm formation by using confocal microscopy. No apparent difference was noted, either between the wild type and YliH- mutant or the *Salmonella* carrying *E. coli yliH* (pBAD *yliH*$^+$) (data not shown).

In this study, we have tried to identify the role of YliH in *Salmonella*, significantly induced at the entry of stationary phase, as assessed by microarray analysis and promoter assay (Table 2 and Fig. 3). Since amino acid sequence analysis failed to suggest the role of YliH, we relied on recent reports advocating the characterization of the gene function. The *yliH* together with *YceP* in *E. coli* was reported to regulate biofilm formation, based on the observation that expression of *yliH* was greatly elevated in biofilm stage relative to exponential-growth planktonic culture (Domka *et al.*, 2006). For *ΔyliH*, the biomass increased 290 fold increased, thickness 2,700 fold, and surface coverage 31 fold over wild type. For *ΔyceP*, the biomass increased 240 fold, thickness 2,800 fold, and surface coverage 16 fold over wild-type. Mutation in these *E. coli* genes resulted in increase in motility. After examining related phenotypes in various conditions, it was proposed that the two proteins regulate biofilm through signal secretion. This includes modulation of the uptake and export of signaling pathways, including quorum sensing and the putative stationary-phase signal, among many others, although biochemical evidence has yet to be provided. Nevertheless, we examined phenotype of YliH mutant *Salmonella* including biofilm formation, motility, and adherence. We found that the *Salmonella* YliH⁻ mutant was less motile and less adherent than wild type. Moreover, the mutation did not seem to affect biofilm formation as no discernable change in biomass formation was noted. However, most interestingly, we found that *E. coli* YliH provided from multicopy plasmid had no discernable effect on *Salmonella*. This suggests that biofilm formation in *Salmonella* might be through a mechanism independent of YliH. Thus, YliH is produced in a large quantity at the entry into stationary phase in ppGpp-dependent manner, but its physiological role has yet to be elucidated.

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