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Role of sigma factor E in regulation of *Salmonella* Agf expression

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ABSTRACT

Expression of thin aggregative fimbriae (Agf) in *Salmonella*, which is responsible for bacterial cell adhesion to surfaces, aggregation, and formation of biofilms, is regulated by a complex mechanism. In order to identify gene(s) involved in the expression of Agf, the Tn*phoA* transposon was introduced into *Salmonella typhimurium* χ8505 for random mutagenesis. Colonies showing a change from wrinkly-rough morphology to the smooth form were screened for candidates. Through multiple selection processes, a mutant, named *S. typhimurium* CK167 was selected as the final candidate. Analyses of the nucleotide sequences of Tn*phoA* insertion site identified the insertion in *rpoE* gene. *S. typhimurium* CK178, a defined *rpoE* deletion mutant on χ8505, exhibited the same colony morphology as seen in CK167. The *S. typhimurium* CK178 strain expressed significantly reduced amounts of AgfD and showed modulated biofilm formation, demonstrating the role of RpoE in AgfD expression. To the best of our knowledge, this is the first report demonstrating that RpoE acts as a regulator in the expression of Agf in *Salmonella*.

1. Introduction

Salmonella, an enteric pathogen, possesses many different types of surface appendages including flagella [1], type 1 fimbriae [2], Pef [3], Lpf [4], and Agf [5]. Surface appendages play an important role in the attachment of *Salmonella* to host cell surface [6]. It has been known that thin aggregative fimbriae (Agf), sometimes called curli, causes adhesion to surfaces, cellular aggregation and biofilm formation in *Salmonella* spp. [7,8]. *Salmonella* exhibits wrinkly and rough morphology on agar media incubated in Agf expressing conditions. Previous studies demonstrated that Agf expressing *Salmonella* take up Congo red dye when grown on agar media containing Congo red dye [9].

It is well known that genes contained in operons of *agfDEFG* and *agfBAC* are involved in the formation of Agf in *Salmonella typhimurium* [7]. Two divergently transcribed operons *agfDEFG* and *agfBAC* are separated by a 521 bp intergenic region (Fig. 1A). The *agfA* gene encodes the major subunit of Agf fimbriae. Transcription of the *agfBA* operon, encoding the structural components, rigorously depends on the AgfD transcription regulator [7]. The expression of AgfD itself is regulated at the transcriptional level by multiple regulators including OmpR [8], RpoS [10], CpxR [11], Crl [12], and H-NS [10]. Although regulation studies for Agf expression in wild-type strain were done by many research groups, it still leaves questions to be solved due to the involvement of so many different environmental factors [8,13]. Isolation of *Salmonella* strains over-expressing Agf constitutively expanded the studies for Agf as a virulence factor or for regulation of the Agf expression [9,13]. One of Agf over-expressing strains, *S. typhimurium* χ4666 that bears a single point mutation in the −44 *agfD* promoter region (designated as P_{agfD}), exhibited wrinkly morphotype regardless of the growth temperature [13,14]. It has been reported that the Agf expression in χ4666 depends on the presence of OmpR [13]. However, the strain expressed Agf constitutively, regardless of the presence or absence of RpoS, an alternative sigma factor [13]. Based on this report, we hypothesized that alternative regulatory mechanism(s) other than RpoS could be involved in the expression of AgfD in Agf over-expressing *Salmonella*. In this study, we have identified a *S. typhimurium* χ8505 (isogenic strain of χ4666) mutant exhibiting deregulated Agf expression through random transposon mutagenesis. The gene inactivated by transposon-induced mutagenesis was identified.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

The strains and plasmids employed in this study are listed in Table 1. *S. typhimurium* cells were grown at 37 °C or 27 °C in normal or NaCl-free Luria–Bertani (LB) broth or LB agar [15]. Antibiotics, when required, were added to the culture medium at the concentrations described previously by Kang et al. [16]. Diaminopimelic acid (DAP) was added (50 μg/ml) for the *asd*$^-$ strains. LB

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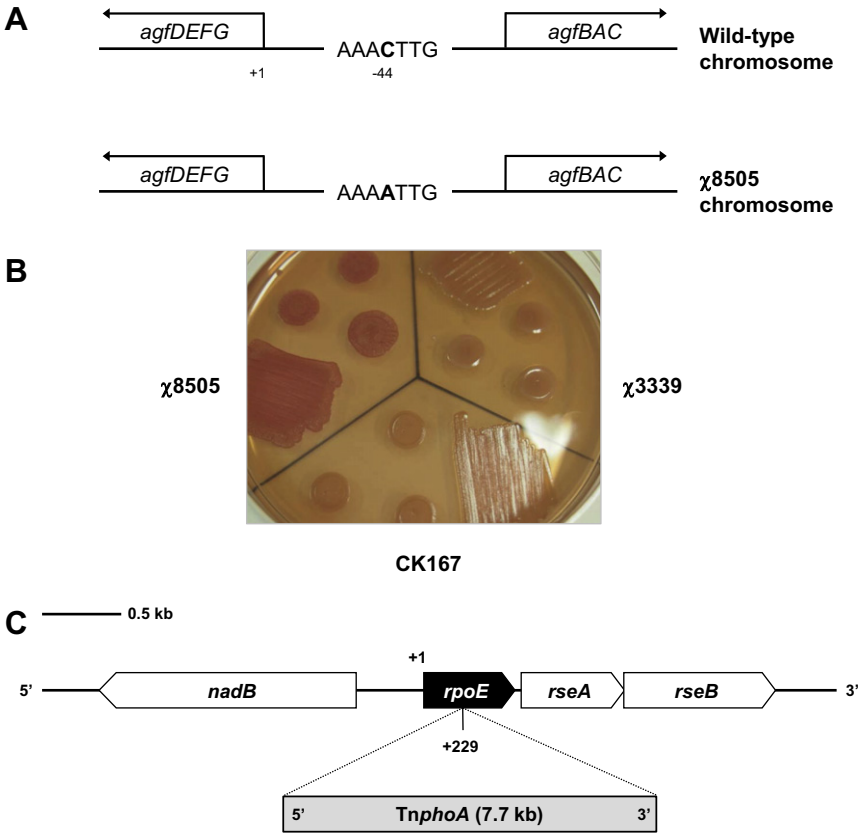

Fig. 1. Gene structure of *agf* operons and *rpoE* region, and wrinkly morphotype of *Salmonella*. (A) Diagram of divergent operons *agfDEFG* and *agfBAC*. Transcriptional orientation of each operon is indicated with arrows. The transcription start site of *agfD* is depicted as +1, and the cytosine residue from the wild-type and the adenine residue from χ8505 at the −44 position [7,13,14] are indicated in boldface. (B) Morphotype changes by transposon insertion. Each *Salmonella* cells were grown on agar plate containing Congo red dye at 27 °C for 48 h. *S. typhimurium strains*; χ3339, wild type; χ8505, constitutive Agf expression (P_{agfD}); CK167, transposon inserted on χ8505. (C) Genetic organization of transposon inserted region in CK167. The *rpoE* gene is indicated with black arrow, and genes around it are indicated with open arrows. Arrows indicate transcriptional orientation of each gene. The 7.7 kb Tn*phoA* transposon insertion site is indicated with dotted lines. The transcription start site of *rpoE* is depicted as +1, and transposon insertion site at the +229 is indicated. Relative sizes of each gene are shown based on a scale bar.

Table 1
Bacterial strains, plasmids, and primers used in this study.

Strains and plasmids	Relevant characteristics	References or sources
Strain		
Escherichia coli		
DH5α	Transformation host for cloning vector, F^-, Φ80d*lac*ZΔM15 Δ(*lacZYA-argF*) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-,m_k^+) *pho*A *sup*E44 l^- thi^{-1} *gyr*A96 *rel*A1	Promega
χ7213	*E. coli* DH5α delivative (*Δasd*), Km^r, DAP required	[21]
Salmonella typhimurium		
χ3339	SL1344, *hisG*, Sm^r, wild type	[25]
χ8505	χ3339 derivative, *PagfD*, constitutive expression of *agfD*	Lab collection
CK167	χ8505 derivative, *PagfD rpoE*::*TnphoA*, Km^r	This study
CK160	χ3339 derivative, *ΔrpoE*	This study
CK178	χ8505 derivative, *ΔrpoE*	This study
CK218	χ8505 derivative, *ΔagfD*	Lab collection
Plasmid		
pRT733	pJM703.1 derivative suicide plasmid carrying *TnphoA*, R6 K *ori*, *mob*, Ap^r, Km^r	[20]
pRE112	Suicide plasmid, R6 K *ori*, Cm^r	[24]
pRK415	Cloning vector, RK-2 *ori*. Tc^r	[28]
pBP675	pRE112 derivative, recombinant suicide plasmid for *ΔrpoE*, Cm^r	This study
pBP711	Full *rpoE orf* in pRK415 for complementation, Tc^r	This study

agar containing 5% sucrose was used for *sacB* gene-based counter-selection in the allelic exchange experiment [17]. An agar media containing dyes Congo red (20 μg/ml) and Coomassie brilliant blue G250 (10 μg/ml) was used to detect Agf binding to dyes [7].

2.2. General DNA manipulations

DNA manipulations were conducted as described by Sambrook and Russel [18]. Transformation into *Escherichia coli* or *Salmonella*

was conducted via either rubidium chloride heat shock or electroporation (BioRad). The recombinant suicide plasmid was conjugally transferred into *Salmonella* using *E. coli* χ7213 (*asd*$^-$) as a plasmid donor. We used PCR amplification to obtain DNA fragments for the cloning and verification of the mutated genes on chromosome.

2.3. TnphoA-mediated transposon mutagenesis and P22-mediated transduction

Tn*phoA* transposon mutagenesis was employed to identify alternative regulators involved in aggregate morphology of *Salmonella* [19]. The pRT733 [20], broad-host-range Tn*phoA* delivery vector, was conjugally transferred from *E. coli* χ7213 [21] to *S. typhimurium* χ8505 (wrinkly and rough morphotype) for random insertion of transposon in the chromosome. Kanamycin-resistant transconjugants exhibiting smooth colonial morphotype on NaCl-free LB agar plates were selected as candidate mutants for alternative regulator(s).

P22 bacteriophage-mediated transduction was employed with the methods described in Orbach et al. [22]. P22 phages raised in smooth type *S. typhimurium* mutants generated by transposon insertion were used for transduction of chromosomal region encompassing transposon to *S. typhimurium* χ8505 [23].

2.4. Construction of a S. typhimurium rpoE deletion mutant

The 0.8 kb 5′-flanking and 0.8 kb 3′-flanking regions of the *rpoE* gene were amplified by PCR from *S. typhimurium* χ3339 chromosomal DNA as a template with use of primer pairs (E1, 5′-CCG GTACCATCATAAGCCACTG-3′; E2, 5′-CCGAATTCCCGAGGTAATGTCT C-3′; E5, 5′-CCGAATTCCGATAGCGGGATACT-3′; E6, 5′-CCGAGCTC-CAAACCAAAGTTGCT-3′). Each flanking DNA, restricted by enzymes, was ligated *in vitro* and subjected to additional PCR using primers E1 and E6, resulting in 1.6 kb recombinant DNA. The 1.6 kb purified PCR product was subcloned into a suicide vector pRE112 [24], resulting in a recombinant suicide plasmid pBP675 (Table 1).

The Δ*rpoE* mutation was introduced in *S. typhimurium* χ3339 [25] and χ8505 by allelic exchange using recombinant suicide plasmid pBP675, yielding *S. typhimurium* CK160 and CK178, respectively. The 0.6 kb *rpoE* gene deletion was confirmed genetically by size comparison of DNA amplified by means of PCR with primers E1 and E6.

2.5. Quantitation of the biofilm formation

Biofilm development was examined by the modified protocol of Römling [26]. *Salmonella* were grown in 96-well microtiter plates containing LB broth with or without NaCl after incubation for 24 h at 37 °C or 27 °C without shaking [26]. Cell clumping and pellicle formation were inspected visually. *Salmonella* adherence to the surface of microtiter plates was qualified by performing crystal violet staining. Adherence was quantified by the amount of dye bound to surface. Bound dyes after washing with trickling water were dissolved in 200 μl of 95% ethanol. The intensity of violet color was measured by determining of optical density at 595 nm. The value of $OD_{595\,nm}$ was normalized by cell density used in the reaction. Experiments were performed thrice for statistical analysis.

2.6. SDS–PAGE and immunoblot analysis

Protein samples solubilized in 2× digestion buffer were boiled for 5 min and separated by discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [18]. The separated proteins were stained with Coomassie brilliant blue G-250 (Sigma), and were destained using a destaining solution.

For immunoblotting, the proteins separated by SDS–PAGE were transferred to nitrocellulose membrane (Whatman) through modified procedures of Towbin et al. [27]. The membranes blocked in 5% skim milk were incubated for 2 h with suitably diluted antisera solution, followed by the treatment of a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) solution. Immunoreactive bands were detected by the addition of 4-chloro-1-naphthol (Sigma) in the presence of H_2O_2 (Sigma). The reaction was stopped by washing with several changes of large volumes of deionized water.

3. Results

3.1. Identification of a S. typhimurium mutant deregulating Agf expression

To identify strains carrying deregulated Agf expression in *S. typhimurium* χ8505, Tn*phoA* transposon mutagenesis was performed. From approximately 100,000 kanamycin-resistant colonies, 80 colonies exhibited smooth (unwrinkled) morphotype on NaCl-free LB agar. Elimination of transconjugants having Tn*phoA* insertion in *agfBAC* and *agfDEFG* operons was required. In order to determine whether the transposon had been inserted in the operons or not, DNA size comparison was performed after PCR amplification of each *agf* operon using several sets of primers (*agfBAC*, 5′-GCGAATTCGGAAGCATAAGAACA-3′ and 5′-GAGCTCCTC AATGATTAGTCATCCT-3′; *agfDE*, 5′-GCGGATCCCCTATCGAAGAGAC G-3′ and 5′-GCGAATTCTGTGTGTTATGCCGGC-3′; *agfEFG*, 5′-GCGG ATCCGCTTTGTCGTATTCA-3′ and 5′-GCAAGCTTCTGCTCTTCGATA GG-3′). Forty-one out of 80 strains contained transposon insertion in *agf* operons: 17 in *agfDEFG* operon and 24 in *agfBAC* operon (data not shown). The remaining 39 mutants were characterized more specifically. To determine the association of Tn*phoA* insertion at specific gene and phenotypic changes of χ8505, chromosomal regions containing Tn*phoA* in 39 mutants were transferred into fresh *S. typhimurium* χ8505 through P22 bacteriophage-mediated transduction. Association of Agf phenotypic changes and kanamycin resistance was assessed on the basis of over 70% of co-transduction frequencies. It had been turned out that most of the mutants (37 mutants) showed co-transduction frequencies below 70%, whereas only 2 strains showed a rate of above 70%. Since previous studies have demonstrated that OmpR is a regulator of Agf expression [8], we decided to determine whether the Tn*phoA* insertion in 2 final candidate mutants is in the *ompR* gene. One mutant carried Tn*phoA* insertion in the *ompR* gene (data not shown). Finally, another remaining mutant was chosen as the final candidate and named *S. typhimurium* CK167. The morphotype of *S. typhimurium* CK167 was examined on agar plates containing Congo red and Coomassie. The strain CK167 exhibited a wrinkle-free morphotype and reduced binding of dyes compared to the parental strain, χ8505, indicating that a gene inactivated by transposon insertion causes decreased expression of Agf (Fig. 1B).

3.2. Identification of a regulatory gene involved in S. typhimurium Agf expression

In order to identify the Tn*phoA* insertion site, chromosomal walking PCR (Seegene) was conducted using several random primers (sequences not shown) and a specific primer for 5′ end of Tn*phoA* (TSP1, 5′-CGGTTGCTGATGCAGCCGAGT-3′; TSP2, 5′-CCCCATCCCATCGCCAATCAGCA-3′; TSP3, 5′-GGTTCCGTCCAG-GACGCTACTTG-3′). The PCR amplified products were sequenced to determine the locus of transposon insertion. Analysis of

nucleotide sequences suggested that Tn*phoA* was inserted in the middle of *rpoE* gene (at 229 nt from start codon) encoding an alternative sigma factor E subunit of RNA polymerase (Fig. 1C). In order to confirm the insertion of Tn*phoA* within the *rpoE* gene, peripheral regions of *rpoE* were PCR amplified using CK167 and χ8505 chromosomal template DNA. All the sizes of amplified DNAs were as expected (data not shown).

3.3. Involvement of rpoE gene in the expression of wrinkly morphotype

For our convenience in conducting experiments, we introduced a defined deletion of *rpoE* gene into *S. typhimurium* χ3339 (wild-type) and χ8505 (χ3339 P_{agfD}) chromosome through the process described in Section 2, resulting in *S. typhimurium* strains CK160 (χ3339 Δ*rpoE*), CK178 (χ8505 Δ*rpoE*), respectively. The morphotype of these stains were characterized on Congo red containing NaCl-free LB agar. In contrast to χ8505 exhibiting wrinkly and rough phenotype on NaCl-free LB agar plate, the *S. typhimurium* CK178 strain formed smooth colonies and showed reduced dye binding (Fig. 2). To examine the phenotype of the RpoE complementing strain, a plasmid pBP711 (Table 1) harboring the entire *rpoE* gene was transformed into CK178. The *S. typhimurium* strain carrying pBP711 reverted to the wrinkly-rough morphotype. The phenotype of CK178 containing PRK415 (vector control) [28] was similar to that of CK178. Since AgfD is a known regulator for the transcription of Agf operon, we examined the contribution of RpoE to AgfD protein expression at the protein level. Anti-AgfD polyclonal antibodies were generated in rabbit, according to the standardized methods used in our laboratory [29]. Lysates of *Salmonella* cells were subjected to Western blotting analysis. The AgfD protein was detected in χ8505 in contrast to χ3339, CK160, and CK218 (χ8505 Δ*agfD*, negative control) (Fig. 3). The *S. typhimurium* CK178 strain expressed significantly reduced amount of AgfD protein (0.3-fold), when compared to the parental strain, χ8505. As expected, high levels of AgfD protein were detected in the CK178 strain bearing multi-copy *rpoE* complementing plasmid pBP711. Nonspecific immune-reactive bands were considered as the loading control. Consistency of morphotype properties and Western blot analysis demonstrates that RpoE, an alternative sigma factor, is involved in the expression of Agf by regulating the expression of the gene that produces the AgfD protein.

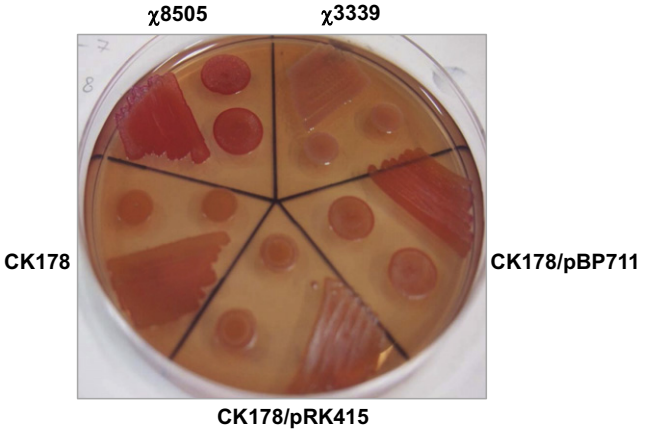

Fig. 2. Morphotype complementation of CK178 by supplying pBP711. Plasmid pBP711 carrying entire *rpoE* gene was transformed into CK178 strain for *rpoE* complementation. As a plasmid control, pRK415 was also transformed into CK178. *Salmonella* strains were streaked on agar plate containing Congo red dye and incubated at 27 °C for 48 h. *S. typhimurium* strains; χ3339, wild type; χ8505, constitutive Agf expression (P_{agfD}); CK178, defined deletion of *rpoE* on χ8505.

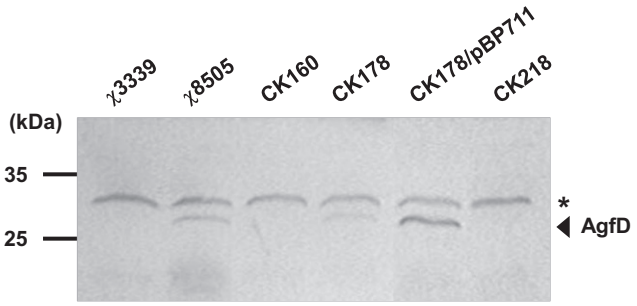

Fig. 3. Western blot analysis to detect AgfD protein. *Salmonella* cells grown at 27 °C in LB agar plates for 48 h were scraped and resuspended in fresh NaCl-free LB broth with vigorous shaking. Cell density of each sample was adjusted by addition of NaCl-free LB broth along with measurement of $OD_{600\,nm}$. Western blot was done by processes described in Section 2. Protein size maker is indicated on left side. Immuno-reactive AgfD protein is indicated on right side. Nonspecific immune-reactive bands (asterisked) were considered as the loading control.

3.4. Contribution of rpoE deletion in biofilm formation

It has been known that Agf is one of the factors involved in biofilm formed by *Salmonella* [6]. Formation of biofilms was quantified by staining the culture container surface with crystal violet after growing *S. typhimurium* strains under various culture conditions. Formation of biofilms was not detectable when the cells of wild type and CK160 derivatives were cultured in LB broth with or without NaCl at 27 °C or 37 °C (Fig. 4). The formation of biofilms from cells of χ8505 and CK178 derivative strains was greatly increased when cells were grown under NaCl-free conditions, regardless of the growth temperature. As seen in Fig. 2, although CK178 has significantly reduced amount of AgfD, it still may allow the transcription of *agf* operons thus accumulating Agf during culture for assay. Interestingly, *S. typhimurium* CK178 cells grown in LB containing NaCl exhibited reduced formation of biofilms at both temperatures 27 °C and 37 °C, when compared to that observed in the case of χ8505. CK178 carrying pBP711 reverted to its phenotype and produced biofilms to the same extent as the χ8505 strains did.

4. Discussion

Many factors that influence the attachments of *Salmonella* have been studied from the point of them being virulence factors [6]. Since hosts show immune reaction to adhesions, pathogens try their best to change the display of adhesion factors (termed as phase variation) to avoid inducing the host immune system reactions [30]. The phase variation of Agf in *Salmonella* is reported to be regulated by a combination of various environmental factors [8,13] and genetic regulators [8,10–12]. Studies of the expression of Agf regulation gained momentum following the characterization of the *S. typhimurium* χ4666 strain [13,14]. *S. typhimurium* χ8505 generated in a previous study is an isogenic strain of χ4666 [31]. The strain exhibited a wrinkly and rough morphotype when grown on agar media [7]. These bacteria adhere very well to dyes such as Congo red [9]. Based on these properties, we conducted this study to identify the regulators of Agf expression in *S. typhimurium* χ8505.

Through random mutagenesis using Tn*phoA* transposon, we selected colonies exhibiting a smooth morphotype amongst more than 100,000 kanamycin resistant colonies. After excluding strains with transposon inserted into *agf* operons, we obtained 39 strains. By P22 bacteriophage mediated co-transduction process, we could see that all the strains except for 2 of them had no connection with transposon insertion. Therefore, we assumed that 37 strains are results of unknown spontaneous mutations. One of the remaining 2 strains was confirmed to be an insertion in *ompR*, and hence, we

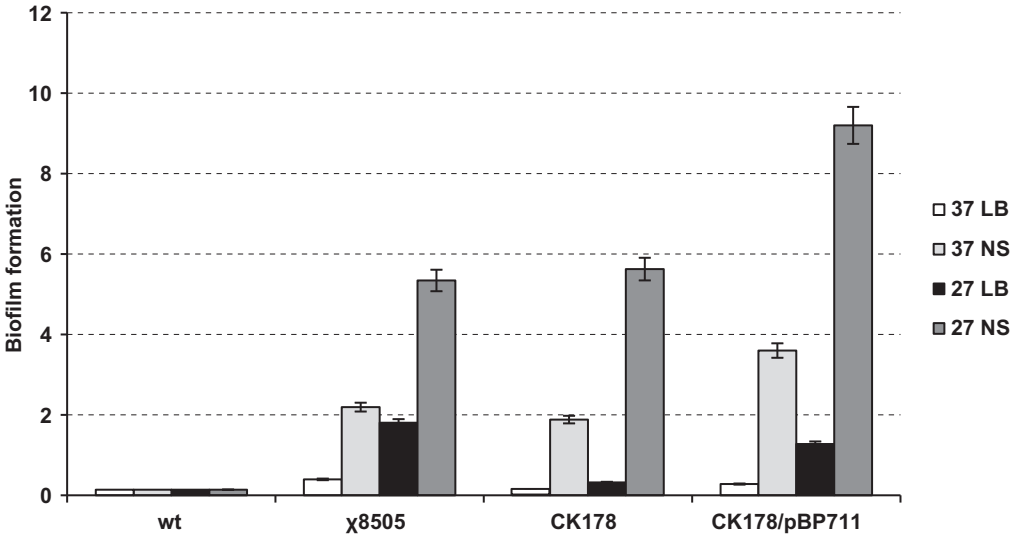

Fig. 4. Assay for biofilm formation. Appropriate amount of pre-cultured *Salmonella* cells were inoculated to 96-well microtiter plates containing LB broth with or without NaCl. A set of plates was incubated at 37 °C for 24 h, and another set was incubated at 27 °C for 48 h. Biofilm formation was quantified by the protocols described in Section 2. The value normalized by initial cell density was presented. Statistically analyzed standard deviation is indicated.

chose the other one to be the final candidate, and named it *S. typhimurium* CK167 (Fig. 1).

To determine whether morphotype change was caused by an occasional polar effect which occurs when transposon insertion is performed or whether it was an effect of a mutated *rpoE* gene, we constructed a mutant strain, CK178 with a defined *rpoE* deletion in the χ8505 strain. Reduced amount of AgfD in CK178 and recovery of AgfD by supplying pBP711 confirmed that RpoE regulates AgfD expression (Fig. 3). The reason why CK178 harboring pBP711 expresses high level of AgfD than χ8505 does might be that the pBP711 is a multi-copy plasmid. These data indicated that the expression of AgfD is proportional to the amount of RpoE.

Although RpoE is involved in the regulation of gene expression, the definitive stimuli are not known. Involvement of RpoE in Agf expression in *Salmonella* needs to be studied under conditions where it regulates the expression of Agf. Therefore, we checked for the formation of biofilms one of the biological functions of Agf. We observed that the formation of biofilms varied quantitatively with the combination of various temperatures and the amount of NaCl in the liquid culture. RpoE was influenced by conditions like the alteration of temperature in the presence of NaCl (Fig. 4), indicating that the stimuli activating the role of RpoE are rather complicated. All the results presented in this study demonstrate that RpoE regulates the expression of Agf in *Salmonella*. To the best of our knowledge, this is the first report that demonstrates the role of RpoE in regulating the expression of Agf. However, this study indicates a gap in the knowledge about the stimuli or environmental factors that can activate RpoE, which can be the focus of subsequent research.

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