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Genomic SELEX Search for Target Promoters under the Control of the PhoQP-RstBA Signal Relay Cascade▽

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RstBA, a two-component regulatory system of *Escherichia coli* with an unidentified regulatory function, is under the control of a Mg^{2+}-sensing PhoQP two-component system. In order to identify the network of transcription regulation downstream of RstBA, we isolated a set of RstA-binding sequences from the *E. coli* genome by using the genomic SELEX system. A gel mobility shift assay indicated the binding of RstA to two SELEX DNA fragments, one including the promoter region of *asr* (acid shock RNA) and another including the promoter for *csgD* (a regulator of the curli operon). Using a DNase I footprinting assay, we determined the RstA-binding sites (RstA boxes) with the consensus sequence TACATNTNGTTACA. Transcription of the *asr* gene was induced 10- to 60-fold either in low-pH (pH 4.5) LB medium or in low-phosphate minimal medium as detected by promoter assay. The acid-induced in vivo transcription of *asr* was reduced after the deletion of *rstA*. In vivo transcription of the *asr* promoter was observed only in the presence of RstA. In agreement with the PhoQP-RstBA network, the addition of Mg^{2+} led to a severe reduction of the *asr* promoter activity, and the disruption of *phoP* also reduced the *asr* promoter activity, albeit to a lesser extent. These observations altogether indicate that RstA is an activator of *asr* transcription. In contrast, transcription of *csgD* was repressed by overexpression of RstA, indicating that RstA is a repressor for *csgD*. With these data taken together, we conclude that the expression of both *asr* and *csgD* is under the direct control of the PhoQP-RstBA signal relay cascade.

The two-component system (TCS) is the most common signal transduction system in bacteria (for a review, see reference 31). A typical TCS consists of two proteins, a membrane-associated sensor with histidine kinase (HK) activity and a cytoplasmic response regulator (RR). In most cases, HK senses an environmental signal(s), self-phosphorylates its own conserved His residue, and then transfers the phosphoryl group onto a specific Asp residue of the cognate RR pair (31, 39). The phosphorylated RR regulates transcription of a set of genes which are needed for a response to the environmental change. *Escherichia coli* contains a total of about 30 TCS pairs (21, 39). To reveal the specificity of signal transduction between HK and RR, we examined self-phosphorylation in vitro of all purified *E. coli* HKs and transphosphorylation of RR by the phosphorylated HKs for all possible HK-RR combinations (39). In most cases, the transphosphorylation took place between cognate HK-RR pairs, but cross talk of transphosphorylation was identified at least for 14 combinations.

The transcriptional response of *E. coli* to external Mg^{2+} ions is under the control of the PhoQP TCS. Mg^{2+} is an essential metal ion and plays important roles in various cellular activities. PhoQ monitors the availability of external Mg^{2+} and, at low levels of Mg^{2+}, phosphorylates PhoP for its activation. Activated PhoP controls transcription of a set of genes for maintenance of Mg^{2+} homeostasis (15, 20, 38). In a Mg^{2+}-limiting environment, the PhoQP system induces the expression of the Mg^{2+} import system, while under conditions of excess Mg^{2+}, the Mg^{2+} import system is turned off. The decrease in the Mg^{2+} level also appears to be a signal for expression of virulence genes in mammalian hosts (7). After the microarray analysis of the target genes under the control of the PhoQP system, we identified the *rstAB* operon, encoding one of the *E. coli* TCSs as one of the PhoQP targets (20), showing a hierarchy for the PhoQP-RstBA regulation network. Up to now, however, neither a specific stimulus for *rstAB* expression nor target genes under the control of the RstBA TCS has been identified (9, 22, 32, 39, 40).

In this study, we tried to search for the RstA-binding sequences by using the newly developed genomic SELEX (systematic evolution of ligands by exponential enrichment) system (27). Once the consensus recognition sequence is predicted after SELEX screening, one can extend the search of other RstA targets against the whole genome sequence. From the SELEX results herein described, two genes are found to be under the control of RstBA TCS: *asr* (acid shock RNA), encoding an acid-inducible protein with unidentified function, and *csgD*, encoding a master regulator that controls the formation of curli fimbriae or bacterial amyloid fibers. Using the newly identified RstA box sequence, we could identify nine other candidates of the RstA target. Several lines of experimental evidence indicated that the expression of *asr* is activated by RstA in response to low Mg^{2+} availability in the

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environment, while that of *csgD* is repressed. We thus propose that PhoQP-RstBA forms a signal relay cascade of the stress response transcription regulation network in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and culture media. For expression and purification of RstA, the expression plasmid pKH13-1 was transformed into BL21(DE3) [F$^-$ *ompT hsdSB* (rB$^-$ mB$^-$) *dcm gal* λ(DE3)]. For expression of RstA, transformants were grown in LB medium at 37°C. For the promoter assay, a TFP (two-fluorescent protein) promoter assay vector was transformed into wild-type *E. coli* KP7600 (W3110 *lacI*q *lacZΔM15 galK2 galT22*) (27), the *phoP*-disrupted mutant JD22184 (KP7600 *phoP*), or the *rstA*-disrupted mutant JD22755 (KP7600 *rstA*). Cells were cultured in LB medium or low-phosphate minimal-glucose medium (LPM) (33) supplemented with peptone (0.6 mg/ml) at either pH 7.0 or pH 4.5. For the single-copy assay of the *asr* promoter, MCasr (MC4100, λ*asr*::*lacZ*) was constructed according to the method of Simons et al. (28). In brief, pRSasr was recombined with phage λRS45, and the recombinant phages were lysogenized into MC4100 to isolate MCasr on an LB agar plate. When necessary, 100 μg/ml ampicillin and 25 μg/ml kanamycin were added to the medium.

Plasmid construction. For the construction of IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible RstA expression plasmid pKH13-1, a DNA fragment corresponding to the RstA-coding region was amplified by PCR using *E. coli* W3110 genome DNA as a template and a pair of primers, RSTAF (5′-CGTTTTATAGGATCCGTGAATGTTATGAA-3′) and RSTAR (5′-CAGTTTTTTCGCGGCCGCTTCCCATGCATG-3′), and, after digestion with BamHI and NotI, cloned into pET21a(+) (Novagen) at the corresponding sites (38). For construction of an arabinose-inducible RstA expression plasmid, a DNA fragment (830 bp) containing the *rstA* coding region was prepared by PCR using *E. coli* KP7600 genome DNA as a template, along with a pair of primers, rstA-BAD-EcoRI-F (5′-TAGAAAGAATTCATAGTAAGTAAAAACAGC-3′) and rstA-BAD-XbaI-R (5′-CAAGGATCTAGACAAACAATAACAGGTAAA-3′). After digestion with EcoRI and XbaI, the PCR-amplified fragment was inserted into pBAD18 (8) at the corresponding site to generate the plasmid pBADrstA.

For quantitative measurement of the promoter activity, the test promoters were inserted into the promoter assay vector pGRP, which carries two fluorescent protein genes, one encoding red fluorescent protein (RFP) and another coding for enhanced green fluorescent protein (eGFP) (18, 26). The RFP gene was under the control of a reference promoter *lac*UV5 while the test promoter was inserted upstream of the GFP coding sequence. Promoter region of the *asr* gene was amplified by PCR using genomic DNA from KP7600 as the template and the pair of primers M021S (5′-GAAGATCTACGCTGGGTGGTGTTTTCTGG-3′) and M021T (5′-CGATGCATTGTCATACCCTCAATTTGTTTTTTCATTTAAC-3′), while the promoter region of the *csgD* gene was amplified by PCR using the pair of primers N020S (5′-ACATGCATGCATGATGAAACCCCGCTTTTTTTATTG-3′) and N020T (5′-GAAGATCTCAGTCATTCTTCTTGCCCGTCG-3′). These primers contain the recognition sequences by EcoT22I, BglII, or BamHI for suitable for cloning. The PCR products were digested with the respective restriction enzymes and then ligated into pGRP at EcoT22I and BglII sites (18, 26). The insertions in the promoter assay plasmids thus constructed were confirmed by sequencing, and the plasmids were named pGRM021 and pGRN020, respectively. For the construction of a single-copy *lacZ* reporter vector, a DNA fragment (354 bp) containing the *asr* promoter region was prepared by PCR using *E. coli* KP7600 genome DNA as a template and a pair of primers, asr-EcoRI-F (5′-GCCAGCGAATTCCCGCAGCGCGTCTAG-3′) and asr-BamHI-R (5′-AGCGGCAGGATCCAGAGCTAATACTTT-3′). After digestion with EcoRI and BamHI, the PCR-amplified fragment was inserted into pRS551 (28) at the corresponding site to generate the plasmid pRSasr.

Purification of RstA and RNA polymerase proteins. His-tagged RstA was expressed in *E. coli* BL21(DE3) containing pKH13-1 and purified as described previously (27, 39). RNA polymerase core enzyme was purified from *E. coli* W3350 (16). Subunit σ^{70} was expressed using pGEMD and purified according to the method used by Igarashi and Ishihama (12). Holoenzymes were reconstituted by mixing the core enzyme and a fourfold molar excess of the σ subunit.

SELEX search for RstA-binding sequences. The genomic SELEX system was used as described previously (27). Genome DNA of *E. coli* W3110 was sonicated to generate fragments of 200 to 300 bp in length. The *E. coli* DNA library was constructed after cloning of these 200- to 300-bp DNA fragments into plasmid pBR322 at the EcoRV site. A collection of 200- to 300-bp DNA fragments could be regenerated by PCR amplification using the *E. coli* DNA plasmid library as the template and a set of primers, EcoRV-F (5′-CTTGGTTATGCCGGTACTGC-3′) and EcoRV-R (5′-GCGATGCTGTCGGAATGGAC-3′), which hybridize to pBR322 vector at EcoRV junctions. PCR products thus generated were purified by 6% polyacrylamide gel electrophoresis (PAGE). For the genomic SELEX screening of RstA-binding sequences, 5 pmol of DNA fragments and 20 pmol of His-tagged RstA were mixed in a binding buffer (10 mM Tris-HCl, pH 7.8 at 4°C, 3 mM Mg acetate, 150 mM NaCl, 1.25 μg/ml bovine serum albumin [BSA]) and incubated for 30 min at 37°C. The mixture was applied onto a Ni-nitrilotriacetic acid column, and after washing unbound DNA with the binding buffer containing 10 mM imidazole, DNA-RstA complexes were eluted with an elution buffer containing 200 mM imidazole. When necessary, this SELEX cycle was repeated several times. For sequencing of RstA-bound DNA fragments, DNA fragments were dissociated and isolated from DNA-RstA complexes by PAGE and PCR amplified. PCR products were cloned into pT7 Blue-T vector (Novagen) using the blunt-end cloning kit (Takara) and transformed into *E. coli* DH5α. Fluorescence-labeled DNA was prepared using primer 2T7P (5′-TAATACGACTCACTATAGGG-3′), and sequencing was performed with ABI DNA sequencer.

Measurement of the promoter activity. Promoter strength was determined as described previously (18, 26). In brief, GFP was expressed under the control of a test promoter while RFP was under the control of a reference promoter. For the measurement of the fluorescence intensity of RFP or GFP expressed in *E. coli*, cells grown in LB medium or LPM up to an optical density at 600 nm (OD_{600}) of 0.6 to 0.8 were harvested by centrifugation, resuspended in phosphate-buffered saline, and diluted with phosphate-buffered saline to obtain approximately the same cell density (OD_{600} = 0.6) for all samples. For the measurement of bulk fluorescence, aliquots of a 0.2-ml cell suspension were added to 96 × 0.4-ml flat-bottom wells, and the fluorescence was measured with a Wallac 1420 ARVOsx (Perkin-Elmer Life Sciences), where GFP was measured using 485-nm excitation and 535-nm emission and RFP was measured using 544-nm excitation and 590-nm emission. The fluorescence intensity of GFP by the test promoter was normalized using the equation $(X/Y)/(A/B)$, in which X and Y indicate the fluorescence intensities of GFP (test promoter) and RFP (*lac*UV5 promoter), respectively, while A and B represent the fluorescence intensities of GFP (*lac*UV5 promoter) and RFP (*lac*UV5 promoter), respectively.

Preparation of labeled probes for gel shift and DNase I footprinting assays. Probes were generated by PCR amplification of the *asr*, *csgD*, *nikA*, *yqaD*, *ptsP*, *ykfG*, *yecP*, and *gntU* promoter regions by using a pair of primers, 5′-fluorescein isothiocyanate (FITC)-labeled FITCT7pro primer (5′-TAATACGACTCACTATAGGG-3′) and T7-R primer (5′-GGTTTTCCCAGTCACACGACG-3′), SELEX fragment-containing plasmids (100 ng) as the template, and Ex *Taq* DNA polymerase (Takara). PCR products with FITC at their termini were purified by PAGE.

Gel shift assay. Each 0.5 pmol of FITC-labeled probe was incubated at 37°C for 30 min with various amounts of RstA in 12.5 μl of gel shift buffer consisting of 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 3 mM Mg acetate, and 25 μg/ml BSA. After the addition of the DNA dye solution (40% glycerol, 0.025% bromophenol blue, 0.025% xylene cyanol), the mixture was directly subjected to 6% PAGE.

DNase I footprinting assay. DNase I footprinting assay was carried out using FITC-labeled DNA fragments as described previously (N. Fujita and A. Ishihama, unpublished data). Each 1.0 pmol of FITC-labeled probes was incubated at 37°C for 30 min with various amounts of RstA in DNase I footprinting buffer consisting of 25 μl of 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 3 mM magnesium acetate, 5 mM $CaCl_2$, and 25 μg/ml BSA. After incubation for 30 min, DNA digestion was initiated by the addition of 5 ng of DNase I (Takara). After digestion for 30 s at 25°C, the reaction was terminated by the addition of 45 μl of DNase I stop solution (20 mM EDTA, 200 mM NaCl, 1% sodium dodecyl sulfate, 0.25 mg/ml yeast tRNA). Digested products were precipitated with ethanol, dissolved in formamide dye solution, and analyzed by electrophoresis on a 6% polyacrylamide gel containing 8 M urea.

In vitro transcription. Single-round transcription in vitro was carried out essentially as described previously (14, 16). In brief, 0.1 pmol of promoter DNA fragment was incubated at 37°C for 10 min in the presence or absence of RstA in transcription buffer (50 mM Tris-HCl [pH 7.8 at 37°C], 3 mM Mg acetate, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 25 μg/ml BSA), and then 0.1 pmol of the reconstituted RNA polymerase σ^{70} holoenzyme was added in a final volume of 35 μl. After incubation at 37°C for 20 min to form open complexes, RNA synthesis was initiated by adding 15 μl of substrate-heparin mixture (0.16 mM each ATP, GTP, and CTP, 0.05 mM UTP, 2 μCi [α-^{32}P]UTP, and 0.2 mg/ml heparin). After 10 min at 37°C, the reaction was terminated by the addition of 50 μl of stop solution (40 mM EDTA and 0.3 mg/ml tRNA). RNA was precipitated with ethanol, dissolved in formamide dye solution, and analyzed by 6% PAGE in the presence of 8 M urea. After electrophoresis, the gel was

TABLE 1. SELEX fragments isolated with RstA[a]

SELEX fragment source	No. of clones	Left gene (direction)[c]	SELEX fragment[b]	Right gene (direction)[c]
Spacer regions	67	*ynfM* (→)	(1672761) S (1673186)	***asr*** (→)
	1	***csgD*** (←)	(1103558) S (1103763)	***csgB*** (→)
	1	*yecN* (→)	(1954402) S (1954686)	***yecP*** (→)
	1	***ykfG*** (←)	(263770) S (264034)	*yafX* (←)
	1	***ptsP*** (←)	(2966842) S (2967114)	*mudH* (←)
	1	*yqaC* (→)	(2786282) S (2786487)	***yqaD*** (→)
	1	*acp* (→)	(4026666) S (4026845)	***nikA*** (→)
Open reading frames	2	***gidB*** (←)	(3711314) S (*gidA*) (3711511)	*mioC* (←)
	1	*ybaQ* (→)	(508347) S (*copA*) (508581)	***ybaS*** (→)
	1	***ychG*** (←)	(1297625) S (*adhE*) (1297805)	***ychE*** (→)
	1	*yneA* (→)	(1607970) S (*yneB*) (1608152)	***yneC*** (→)
	1	*fli* (→)	(2018948) S (*fliI*) (2019155)	***fliJ*** (→)
	1	*ypeC* (→)	(2517937) S (*mntH*) (2518188)	*nupC* (←)
	1	*yhgN* (→)	(4064113) S (*gntU*) (4064326)	*gntK* (←)
	1	*yhgI* (→)	(4092778) S (*gntT*) (4092955)	*malQ* (←)
	1	*phoR* (→)	(419756) S (*brnQ*) (419982)	***proY*** (→)
	1	*ubiA* (→)	(4258372) S (*plsB*) (4258565)	***dgkA*** (→)
	1	*nrfG* (→)	(4298380) S (*gltP*) (4298638)	*yjcO* (←)
	1	*sdhA* (→)	(758099) S (*sdhB*) (758393)	***sucA*** (→)

[a] A total of 86 DNA fragments have been isolated by the genomic SELEX and sequenced. Based on the location on the genome, these SELEX fragments could be classified into two groups. Group A fragments contain the sequences from 7 different spacer regions between two neighboring genes, while group B fragments contain portions of 12 different protein-coding sequences.

[b] S, SELEX. The numbers on both sides of each fragment represent the boundary nucleotides along the revised *E. coli* genome (23).

[c] The directions of transcription of the neighboring genes are shown by arrows. The genes shown in bold represent the predicted targets by RstA.

dried and exposed onto an image plate, and then the image plate was analyzed with BAS1000 (Fuji).

Isolation of total RNA. For preparation of total RNA for the primer extension analysis, overnight cultures were diluted 100-fold in 100 ml of LB medium and cells were grown to an OD_{600} of 0.6 to 0.8 (log phase) or 1.6 to 1.8 (stationary phase) in the presence of 0.2% arabinose. RNA purification was carried out as described previously (38).

Primer extension analysis. Primer extension analysis was performed using fluorescence-labeled probes according to the protocol of Yamada et al. (36). In brief, 40 μg of total RNA and 1 pmol of 5′-FITC-labeled primer were mixed in 20 μl of 10 mM Tris-HCl (pH 8.3 at 37°C), 50 mM KCl, 5 mM $MgCl_2$, 1 mM each of dATP, dTTP, dGTP, and dCTP, and 20 U of RNase inhibitor. 5′-FITC-labeled primers used were as follows: 5′-GCGGCAACAACCAGAGCTAA-3′ for *asr* and 5′-GCACTGCTGTGTGTAGTAAT-3′ for *csgD*. The primer extension reaction was initiated by the addition of 5 U of avian myeloblastosis virus reverse transcriptase. After incubation for 1 h at 50°C, DNA was extracted with phenol, precipitated with ethanol, and subjected to electrophoresis on a 6% polyacrylamide sequencing gel containing 8 M urea. After electrophoresis, gels were dried and subjected to autoradiography using DSQ-500 (Shimadzu).

RESULTS

Isolation of RstA-binding sequences by genomic SELEX. For isolation of DNA sequences that are recognized by *E. coli* RstA protein, we employed the genomic SELEX system (27), in which a complete library of genome DNA fragments (100 to 300 bp in length) is used as DNA substrates in place of a mixture of synthetic oligonucleotides with all possible sequences (29). A fourfold molar excess of purified His-tagged RstA protein (20 pmol) was mixed with the DNA fragments (5 pmol), and RstA-associated DNA fragments were recovered as complexes. In the initial stage of SELEX, RstA-bound DNA formed a smear on PAGE, but after five cycles of SELEX, several discrete bands were identified, indicating that some DNA fragments with high affinity to RstA were enriched. These DNA fragments were recovered from the gel and cloned into pT7 Blue-T plasmid (Novagen) for sequencing. A total of 86 independent clones were isolated and could be classified into two different sets after sequencing of the inserts. A total of 73 group A clones carried unique sequences from seven different spacer regions on the *E. coli* genome (Table 1), while the rest of the clones, 13 group B clones, carried portions of 12 different protein-coding sequences (Table 1).

Among 73 group A clones, 67 carried nine different segments, ranging from 149 to 285 bp in length, from a 426-bp-long region between nucleotides 1672761 and 1673186 in the revised *E. coli* genome (23), which is located downstream of *ynfM* (encoding a predicted multipass inner membrane protein) and upstream of *asr* (encoding an acid-stress-inducible protein) (Fig. 1). RstA might have a strong affinity to this

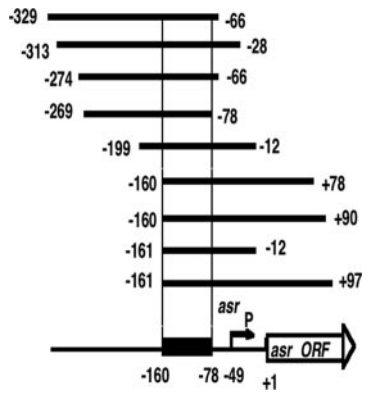

FIG. 1. Alignment of SELEX DNA fragments containing the *asr* promoter (P) region. Thick bars represent the location of SELEX fragments along the sequence upstream from the *asr* gene. The numbers on each line represent the distance (bp) from the initiation codon of the *asr* gene. The sequences between −160 and −78 are conserved among all the SELEX fragments. ORF, open reading frame.

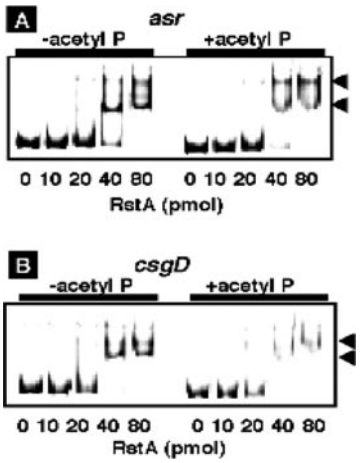

FIG. 2. Gel shift assay. Fluorescence-labeled DNA probes of *asr* (A) or *csgD* (B) were incubated at 37°C for 30 min with the indicated amount (0, 10, 20, 40, and 80 pmol) of RstA in the absence or presence of acetyl phosphate. RstA-DNA mixtures were subjected to PAGE.

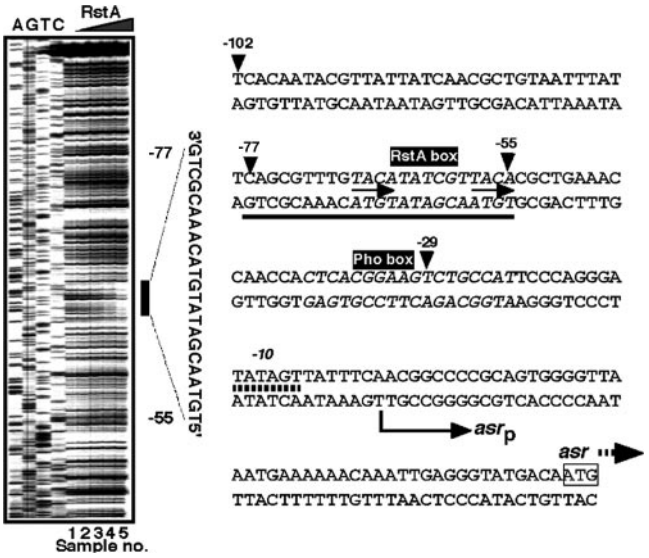

FIG. 3. DNase I footprinting assay of the *asr* promoter. Fluorescence-labeled DNA probe of the *asr* promoter fragment was incubated with increasing concentrations of the purified RstA (lane 1, 0 pmol; lane 2, 10 pmol; lane 3, 20 pmol; lane 4, 40 pmol; lane 5, 80 pmol) and subjected to DNase I footprinting assays as described in Materials and Methods. Lanes A, T, G, and C represent the respective sequence ladders. The bold line under the sequence indicates the RstA-binding sequence as detected by the protection pattern from DNase treatment, while two arrows between the DNA strands indicate the conserved direct repeat of the RstA box. The dotted line between two DNA strands shows the predicted promoter −10 sequence for σ^{70}. The initiation site and direction of transcription are indicated by asr_p. The nucleotide number represents the distance from the transcription initiation site.

ynfM-asr spacer region because the number of SELEX isolates correlates with their affinity to the test DNA-binding protein (27). From the alignment of these nine different SELEX fragments, shown in Fig. 1, the binding site of RstA was predicted to locate within a short overlapping 83-bp segment between positions −160 and −78 from the initiation codon of the *asr* gene. Aside from this *ynfM-asr* segment, only a single clone was obtained from each of other six different spacer regions.

Two group B clones contained the same SELEX fragment with the sequence between nucleotides 3711314 and 3711511 from the *gidA* gene encoding glucose-inhibited division protein A (methyltransferase GidA subunit), which is located downstream of *mioC* (FMN [flavin mononucleotide]-binding biotin synthase subunit) and upstream of *gidB* (encoding glucose-inhibited division protein B; methyltransferase GidB subunit). Interestingly the *oriC* locus for the origin of genome replication exists between this predicted RstA-binding sequence and the *mioC* gene, raising a possibility that RstA controls the *oriC* function. Except for this SELEX fragment from the *gidA* coding sequence, only a single clone was obtained for the other 11 SELEX fragments from 11 different coding sequences of the *E. coli* genome.

Identification of RstA-binding activity for SELEX DNA fragments. For confirmation of RstA binding to these putative RstA-binding DNA sequences identified by SELEX, we next performed the gel shift assay for nine DNA sequences, all seven from group A and two (*gidB*-[*gidA*]-*mioC* and *yhgN*-[*gntU*]-*gntK* [the bracketed genes include the SELEX fragments]) from group B (Table 1). The *ynfM-asr* group A DNA fragment formed two RstA-complex bands on PAGE (Fig. 2A). The location of the RstA-binding site and the direction of transcription of two neighboring genes suggested the possibility that RstA regulates transcription of the *asr* gene (Table 1 and Fig. 1), which is known to be highly induced upon exposure to acidic conditions (pH < 5.0) (25, 33).

Besides this *asr* upstream sequence, the spacer sequence between the *csgD* and *csgB* genes also formed two RstA-complex bands (Fig. 2B). This RstA site is located upstream of both of the neighboring genes, *csgD* (regulator of the *csgBA* operon) and *csgB* (minor curlin subunit) (Table 1). From the location of this RstA site, as determined by the DNase I footprinting assay (see below), RstA bound on this site was considered to regulate the *csgD* gene encoding a FixJ family transcription factor, which positively regulates the *csgBA* operon encoding the minor and major subunits of curli (11).

The RR of TCS is activated in vivo after phosphorylation by the cognate sensor HK (31), but it can be activated in vitro by phosphorylation with acetyl phosphate (39). When the gel shift assay of RstA was carried out in the presence of acetyl phosphate, however, little enhancement of its affinity to the target DNA was observed (Fig. 2). This finding is in agreement with our previous observation that the level of transphosphorylation in vitro of RstA by phosphorylated RstB is lower than that for other RRs of *E. coli* TCSs or that the dephosphorylation activity of phosphorylated RstA is higher than that for other RRs of *E. coli* TCSs (37).

In contrast to these two SELEX fragments, no significant gel shift was observed for the five other group A SELEX fragments and two group B SELEX fragments under the gel shift conditions employed (data not shown). Stable binding of RstA to these sequences might require an effector for RstA activation or its fully phosphorylated state. However, the possibility that these DNA fragments represent nonspecific backgrounds in the genomic SELEX screening is not excluded.

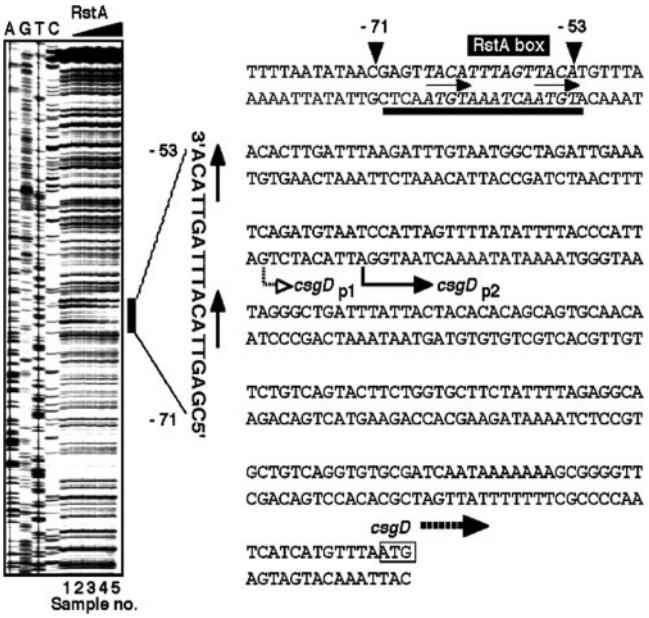

FIG. 4. DNase I footprinting assay of the *csgD* promoter. Fluorescence-labeled DNA probe of the *csgD* promoter fragment was incubated with increasing concentrations of the purified RstA (lane 1, 0 pmol; lane 2, 10 pmol; lane 3, 20 pmol; lane 4, 40 pmol; lane 5, 80 pmol) and subjected to DNase I footprinting assays as described in Materials and Methods. Lanes A, T, G, and C represent the respective sequence ladders. The bold line under the sequence indicates the RstA-binding sequence as detected by protection pattern from DNase treatment while two arrows between the DNA strands indicate the conserved direct repeat of the RstA box. The transcription start site herein identified (Fig. 8) is indicated with *csgD* P2, which is 10 bp downstream from the previously reported promoter P1 (11). The nucleotide number represents the distance from the newly identified transcription initiation site P2.

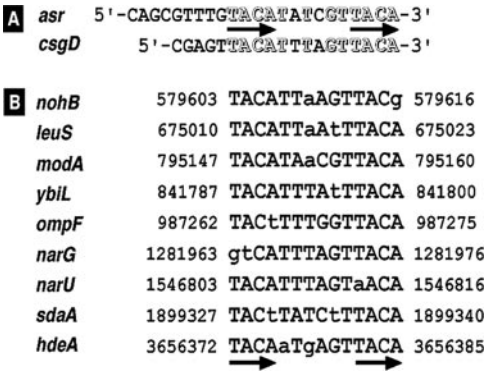

FIG. 5. The consensus sequence of the RstA-binding site. (A) The binding sites of *asr* and *csgD* have been determined in this study. The common bases between these two sequences are shown in outlined, white letters. The arrows indicate a direct repeat located at both termini of the common sequences. The RstA box is shown in bold. (B) After we performed a search for the RstA-binding common sequence in the whole *E. coli* genome, an additional nine sites were identified, all located near the promoters of the indicated genes.

Identification of RstA-binding site on the *asr* and *csgD* promoter regions. To identify the RstA-binding site on the *asr* and *csgD* promoter regions, we next performed the DNase I footprinting assay. On the *asr* promoter fragment, RstA was found to bind to the 23-bp-long sequence between positions −77 and −55 upstream of the *asr* transcriptional start position (or between positions −126 and −104 from the translation start site) (Fig. 3). This RstA-binding sequence is included in the overlapping 83-bp sequence included in all nine SELEX clones (Fig. 1). On the other hand, on the *csgD* promoter fragment, RstA bound to a 19-bp sequence between positions −71 and −53 upstream of the transcriptional start position of *csgD* promoter P2 (or between positions −61 and −43 of promoter P1) (Fig. 4). In the two RstA-binding sequences on the *asr* and *csgD* promoters, a 14-bp-long TACATNTNGTTACA sequence (Fig. 5A), which includes a direct TACA repeat, exists (this sequence is hereafter referred to as the RstA box).

After searching for this RstA box-like sequence along the entire *E. coli* genome, we found nine other possible targets of RstA, including the *nohB*, *leuS*, *modA*, *ybiL*, *ompF*, *narG*, *narU*, *sdaA*, and *hdeA* genes, each carrying the RstA box sequence within 500 bp upstream of the respective initiation codon (Fig. 5B), among which the expression level of *narG* has been reported to decrease in an *rstAB*-deficient strain (22), while the overexpression of RstA represses *ompF* transcription (H. Aiba, personal communication). These observations altogether raise a possibility that RstA is a typical bifunctional regulator, acting as both an activator and a repressor.

RstA-dependent activation of *asr* transcription. Previous studies indicated that the *rstAB* operon is regulated by the PhoQP TCS, which monitors the availability of extracellular Mg^{2+} (20, 38). To examine PhoQP and RstBA dependency of *asr* transcription, we first employed the promoter assay system using the TFP vector (18, 26). The DNA fragment including the *asr* promoter was inserted into the TFP vector to adjust the *asr* initiation codon to that of GFP. Under the normal growth conditions (pH 7.0), the activity of *asr* promoter was very low in all the test strains (Fig. 6A, lanes 1, 3, 5, and 7), but under the acidic conditions (pH 4.5), the *asr* promoter-driven GFP activity markedly increased (more than 60-fold) in the wild-type strain (Fig. 6A, compare lanes 1 and 2). In the mutant lacking RstA, the acid-induced activation of the *asr* promoter was completely abolished (Fig. 6A, compare lanes 2 and 8), indicating that RstA is indeed involved in the activation of the *asr* promoter.

By adding Mg^{2+} to the wild-type culture, we found that the *asr* promoter activity markedly decreased (Fig. 6A, compare lanes 2 and 4), in good agreement with the previous finding that the expression of *rstAB* is activated by phosphorylated PhoP and the PhoP activation does not take place in the presence of Mg^{2+} (20, 38). Accordingly, the *asr* promoter activity was significantly reduced in the mutant lacking PhoP (Fig. 6A, compare lanes 2 and 6). It is, however, noteworthy that in the *phoP* deletion mutant, the acid-induced activation of *asr* promoter took place, albeit at lower levels than in the wild-type parent (Fig. 6A, compare lanes 5 and 6), because a low level of *rstA* expression took place in the *phoPQ* null mutant (K. Yamamoto, unpublished observation). The overall pattern of the *asr* promoter activity in LPM culture (Fig. 6C) is essentially the same as that of LB (rich-medium) culture (Fig. 6A). Again, virtually no *asr* activity was detected in the *rstA* knockout mutant (Fig. 6B, lanes 7 and 8), supporting the pre-

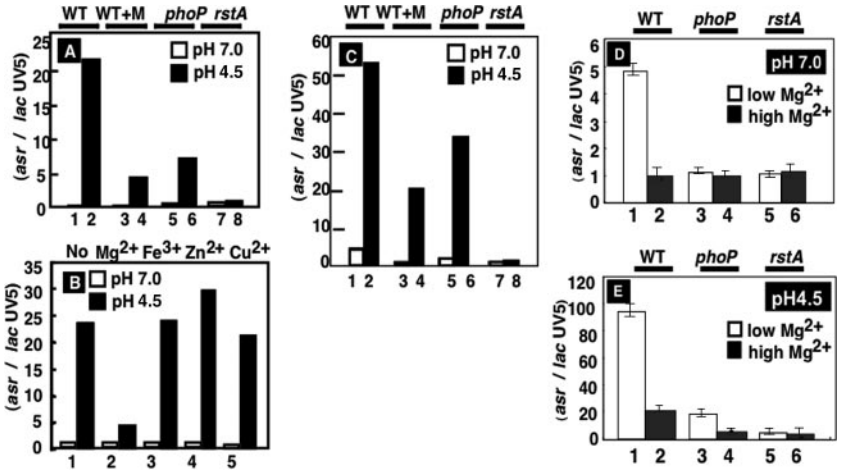

FIG. 6. Influence of *phoP* or *rstA* deletion on the *asr* promoter activity. The *asr* promoter fragment was inserted into the TFP promoter assay vector, and the resulting promoter plasmid was transformed into KP7600 wild type (WT) and its *phoP* or *rstA* deletion strain. The transformants were grown in LB medium or LPM at pH 7.0 or pH 4.5. The promoter activity was determined from the GFP/RFP ratio. (A) The *asr* promoter in LB culture; (B) the *asr* promoter in LB culture containing the indicated metal ions. WT + M, wild-type culture plus Mg^{2+}. (C) The activity of the *asr* promoter was also measured for the wild type and the *phoP* and *rstA* mutants grown in LPM culture. The activity of *asr* promoter was examined for the same set of *E. coli* strains grown in LPM medium at low (0.24 mM) and high (30 mM) Mg^{2+} concentrations and under both neutral (pH 7.0) (D) and acidic (pH 4.5) (E) conditions.

diction that RstA is needed for transcription activation of the *asr* gene.

Influence of RstA binding on transcription of the *asr* gene was also examined using an in vitro transcription system. Here we used truncated templates containing either the *asr* promoter as the test promoter or the reference promoter *lac*UV5. The 350-bp *asr* promoter fragment should produce 74 nucleotides of RNA while the 205-bp *lac*UV5 promoter fragment generates 63 nucleotides of RNA. Transcripts of expected sizes were identified using RNA polymerase $E\sigma^{70}$ holoenzyme (Fig. 7). The activity of the *asr* promoter was low in the absence of RstA, but in the presence of added RstA, it increased in a dose-dependent manner. On the other hand, the activity of *lac*UV5 promoter used as a reference stayed constant irrespective of the presence or absence of RstA. The *asr* promoter was recognized and transcribed by $E\sigma^{70}$ holoenzyme, but transcript was not detected with use of $E\sigma^{38}$ holoenzyme (data not shown).

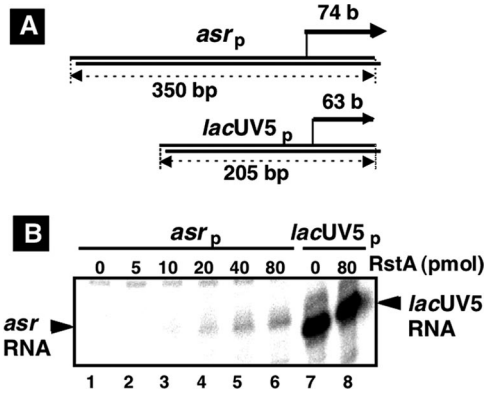

FIG. 7. RstA-dependent transcription in vitro of the *asr* gene. (A) Truncated linear DNA template (350 bp) containing the *asr* promoter was prepared by PCR using pRSasr as the template, asr-EcoRI-F and asr-BamHI-R as primers, and Ex *Taq* DNA polymerase (Takara) and purified by PAGE. Truncated template (205 bp) containing the *lac*UV5 promoter was prepared after digestion of pKB252 (14) with EcoRI followed by PAGE purification. (B) Single-round transcription in vitro was carried out essentially as described in Materials and Methods. Mixtures of promoter fragments and various concentrations of RstA were incubated at 37°C for 10 min, and then the reconstituted RNA polymerase σ^{70} holoenzyme was added and incubated at 37°C for 20 min to allow open complex formation. RNA synthesis was initiated by adding substrate-heparin mixture. RNA synthesis was carried out for 10 min.

Effect of Mg^{2+} addition on acid-induced *asr* expression. The Mg^{2+}-sensing PhoQP TCS forms a signal transduction network with the RstBA TCS (15, 20, 38). Likewise, others reported that the PhoQP TCS forms signal transduction networks with both the iron-responsive BasSR TCS (7, 9) and the divalent cation (Mg^{2+} and Zn^{2+})-responsive three-component system RcsC-YojN-RcsB (9). Therefore, we examined possible influence of Mg^{2+} and other related metal ions to the expression of *asr* under the acidic condition. As shown in Fig. 6B, the acidic pH-induced activation of *asr* promoter markedly decreased by the addition of 30 mM $MgCl_2$, but the other metal ions (Fe^{3+}, Zn^{2+}, and Cu^{2+}) had no significant effect on the *asr* promoter, indicating that neither the BasSR TCS nor the RcsC-YojN-TcsB is involved in *asr* regulation. From the data taken together, we conclude that the acid-responsive *asr* promoter is under the direct control of PhoQP-RstBA signal relay cascade.

To confirm the involvement of both PhoP and RstA in acid-induced transcription of the *asr* gene, the promoter assay was performed in the presence of low (0.24 mM) or high (30 mM) concentrations of Mg^{2+} and at both pH 7.0 (Fig. 6D) and pH 4.5 (Fig. 6E). The *asr* promoter in wild-type *E. coli* was activated at a low level of Mg^{2+} (compare lanes 1 and 2 for both Fig. 6D and E), but the level of its activation was markedly higher at pH 4.5 than at pH 7.0 (compare Fig. 6D, lane 1, and

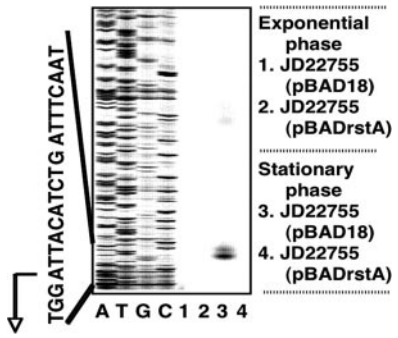

FIG. 8. Primer extension analysis of *csgD* mRNA. *E. coli rstA* mutant JD22755 (pBAD18, control plasmid) (lanes 1 and 3) or JD22755 (pBADrstA, RstA expression plasmid) (lanes 2 and 4) were grown in LB medium. Total RNAs were prepared at either exponential growth phase (lanes 1 and 2) or stationary phase (lanes 3 and 4) and subjected to primer extension assay as described in Materials and Methods. The newly identified transcription start site, shown as promoter P2, is located 10 bp downstream from the previously reported promoter, designated P1 (11) (for the sequence, see Fig. 4).

E, lane 1). The acid-induced activation of the *asr* promoter in the *phoP* mutant (lanes 3 and 4 in both Fig. 6D and E) was as low as that in the *rstA* mutant (lanes 5 and 6 for both Fig. 6D and E), indicating the direct involvement of PhoP in acid-induced activation of the *asr* promoter.

RstA-dependent repression of *csgD* transcription. Since the *csgD* promoter was weaker than the *asr* promoter, as measured by using the TFP promoter assay vector, we then employed the primer extension assay. The influence of RstA on the *csgD* promoter was then examined by direct measurement of *csgD* mRNA after increasing expression of RstA in the *rstA*-null mutant. Total RNAs were prepared from both exponential-phase and stationary-phase cells and subjected to primer extension assay. As shown in Fig. 8, *csgD* mRNA was detected in stationary-phase cells, indicating that *csgD* is one of the stationary-phase-specific genes. The transcription start point herein identified was 10-bp downstream from that published by Hammar et al. (11) (the newly identified *csgD* promoter was designated P2; see Fig. 4 for the sequence). The synthesis of *csgD* mRNA is, however, repressed in cells overproducing RstA protein even though the RstA-binding site is located between positions −71 and −53 upstream from P2 promoter (Fig. 4). Taken together with the location of RstA-binding site on the *csgD* promoter, we propose that transcription of the *csgD* gene is negatively regulated by RstA as in the case of *rpoS* encoding the stationary-phase σ^S subunit of RNA polymerase (32).

DISCUSSION

The acid tolerance response is an important adaptive system for *E. coli* to survive upon exposure to low pH (2). Over 50 acid shock proteins (ASPs) are induced under the acidic conditions, and these ASPs together play roles in protection from the potentially lethal intracellular acidification (2). Since a number of regulatory proteins, including RpoS, Fur, PhoPQ, Ada, PhoBR, EvgAS, GadE, and YdeO, have been reported to be involved in expression of the acid survival system (2, 19, 33), transcription regulations of the ASP genes must be different. The molecular mechanisms underlying acid-pH sensing and transcription regulation of each ASP gene remain largely unsolved.

Using the genomic SELEX system, we identified two target genes (*asr* and *csgD*) regulated by RstBA TCS. The *asr* gene is known to be induced by low pH (<5.0), and the Asr protein is considered to play a role in establishing an acid-tolerant state (24, 33). In this study, we demonstrated, for the first time, that the *asr* gene is under the control of the PhoQP-RstBA transcription factor cascade (Fig. 9). The RstA-binding consensus sequence, the RstA box, was identified on both the *asr* and *csgD* promoters. On the *asr* promoter, the RstA box exists between positions −68 and −55 upstream of the *asr* transcriptional start position (Fig. 3B). Based on the site of RstA binding, it can be predicted that it serves as a class I transcription factor, which makes contact with the C-terminal domain for supporting RNA polymerase binding to the *asr* promoter. RstA homologs have been identified in a number of enterobacterial species. Sequence alignment of the *asr* promoters from different enterobacterial species identified a highly conserved region located at positions −70 to −30 relative to the *asr* transcriptional start site, and after deletion analysis of various segments of this region within the *E. coli asr* promoter, it was shown that sequences upstream from the −40 position were

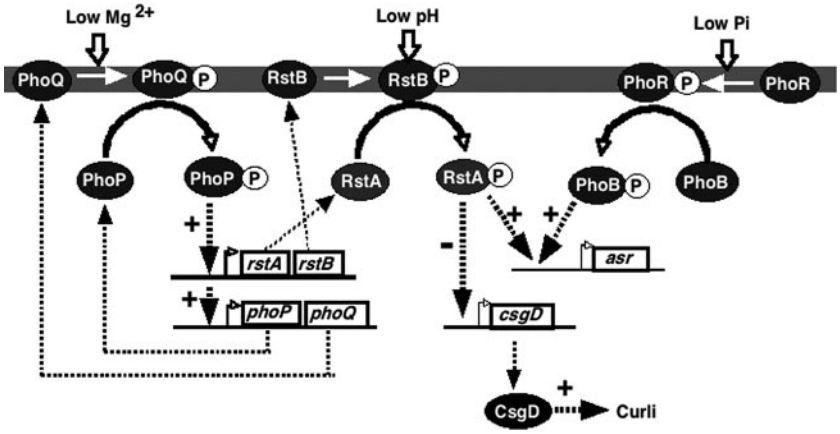

FIG. 9. Model of the PhoPQ-RstAB signal relay cascade for transcription regulation of the stress response genes. Pi, inorganic phosphate.

important for acid induction (25). Thus, the RstA-binding site is located close to the site for acid-pH sensing.

The expression of *asr* is also induced by phosphate starvation and thus depends on the PhoRB TCS (33) (Fig. 9). In fact, the level of *asr* promoter activity in low-phosphate LPM is higher than that in ordinary LB medium (Fig. 6C). The PhoB box is located downstream of the RstA box and overlaps with the promoter −35 sequence (Fig. 3). The PhoQP TCS regulates the transcription of a number of genes in response to changes in extracellular divalent cation concentrations and low pH (2, 17). In *Salmonella enterica* serovar Typhimurium, low-pH environments trigger activation of PhoP even at high Mg^{2+} concentrations (2). Taking the data together, we conclude that the *asr* promoter is under the control of both the PhoQP-RstBA cascade and the PhoRB TCS, each playing a certain role in acid sensing (Fig. 9). In addition, the PmrBA TCS, one of the PhoQP TCS regulation targets, was reported to sense mild acidity in the environment even in the absence of PhoP (30), suggesting that PmrA is also a sensor of low-level acidity. Likewise the iron-responsive BasSR TCS was also reported to be associated with the *E. coli* response to mild acidic conditions (10).

Curli fimbriae (or bacterial amyloid fibers) are produced by many members of the *Enterobacteriaceae* and play important roles in biofilm formation, host cell adhesion, and invasion (1, 4, 5, 28). CsgD is involved in control of expression of a number of genes for curli formation. Reflecting such situations, expression of the *csgD* gene is known to be positively or negatively regulated by a number of transcription factors, including MlrA (5), Crl (3), RpoS (3), integration host factor (6), Rcs (34), the EnvZ-OmpR TCS (13, 35) and the CpxAR TCS (13). In this study, we identified that the expression of *csgD* is also under the control of PhoQP-RstBA cascade. On the *csgD* promoter, the binding site of RstA (RstA box between positions −66 and −53 from P2 [or between −56 and −43 from P1]) completely overlaps with the binding site of OmpR (OmpR box between positions −59 and −40 from P1) (13). The OmpR-EnvZ TCS responds to changes in osmolarity and positively regulates *csgD* expression (13, 35), and thus the expression of *csgD* is activated by the EnvZ-OmpR TCS under high-osmolarity conditions. Here we found that transcription of *csgD* is repressed by overexpression of RstA (Fig. 8). Since OmpR and RstA must compete with each other in binding to the *csgD* promoter, the repression of *csgD* by RstA must be due to competitive inhibition of OmpR binding to the *csgD* promoter. Under certain cases, however, the upstream-bound transcription factors have been shown to repress transcription initiation by preventing the promoter escape of RNA polymerase due to tight interaction of these factors with RNA polymerase (36, 37).

Recently, Barnhart et al. (1) indicated that deletion of the *N*-acetylglucosamine-6-phosphate deacetylase gene, *nagA*, resulted in decreased transcription of the genes *csgBA* and *csgDEFG* encoding curli components and the corresponding decrease in curli production in *E. coli*. The *nagA* gene expression is positively regulated by the PhoPQ TCS under low-Mg^{2+} conditions. These observations altogether indicate that PhoQP is directly involved in positive regulation of curli formation on one hand, and on the other hand, we found that the *csgD* expression is negatively regulated by RstA, implying that the curli formation is under negative regulation by PhoQP-RstBA relay. Involvement of various transcription factors can thus be considered to be a regulation system characteristic of stress response genes in bacteria, monitoring various stress factors and conditions in nature.

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