Screening of promoter-specific transcription factors: multiple regulators for the \textit{sdiA} gene involved in cell division control and quorum sensing

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Prokaryotic DNA-binding transcription factors (TFs) bind in close vicinity of the promoter and regulate transcription through interplay with the DNA-dependent RNA polymerase. Promoters associated with the genes involved in stress response have recently been found to be under the control of multiple regulators, each monitoring one specific environmental condition or factor. In order to identify TFs involved in regulation of one specific promoter, we have developed a PS-TF (promoter-specific TF) screening system, in which the binding of purified TFs to a test promoter was analysed by gel-shift assay. This PS-TF screening system was applied for detection of TFs involved in regulation of the promoter for the \textit{Escherichia coli} \textit{sdiA} gene encoding the master regulator of cell division and quorum sensing. After screening of a total of 191 purified TFs (two-thirds of the predicted \textit{E. coli} TFs), at least 15 TFs have been identified to bind to the \textit{sdiA} promoter, including five two-component system (TCS) regulators, ArcA, CpxR, OmpR, RcsB and TorR. In this study, we focus on these five TFs for detailed analysis of their regulatory roles in vivo. Under normal growth conditions in LB medium, all these TFs repressed the \textit{sdiA} promoter and the repression levels correlated with their intracellular levels. Taken together, we propose that these TCS regulators repress transcription \textit{in vivo} of the \textit{sdiA} gene, ultimately leading to suppression of cell division.

INTRODUCTION

Quorum sensing (QS) is a bacterial cell–cell communication process by which bacteria communicate with each other using extracellular signals called autoinducers (AIs) (Bassler, 2002; Ahmer, 2004). In the bacterial kingdom, three QS systems have been identified, which differ in the molecular species of QS signals. Gram-negative bacteria use \textit{N}-acylhomoserine lactone (AHL) signals, referred to as autoinducer-1 (AI-1), to monitor their own population density and as the language for intra-species communication (Fuqua \textit{et al.}, 1996). The LuxI-family proteins are responsible for production of the AHL autoinducer while the LuxR family proteins play a role in monitoring AHL signals (Waters & Bassler 2005). The model prokaryote \textit{Escherichia coli} K-12 is, however, unable to synthesize AHL molecules because it lacks an AHL-synthase-encoding LuxI-type gene. Nevertheless, \textit{E. coli} carries a predicted AHL receptor of the LuxR family, named SdiA (suppressor of cell division inhibitor) (Garcia-Lara \textit{et al.}, 1996; Michael \textit{et al.}, 2001; Lee \textit{et al.}, 2007). SdiA was originally identified as a regulator of the \textit{ftsQAZ} operon that encodes the division assembly protein FtsQ, the protein FtsA for recruitment of FtsZ to the Z ring, and the tubulin-like cell division protein FtsZ (Wang \textit{et al.}, 1991: de la Fuente \textit{et al.}, 2001; Weiss, 2004; Yao, 2006). The \textit{ftsQAZ} operon carries four promoters, of which the gearbox P1 promoter is transcribed by both RNA polymerase holoenzymes containing RpoD and RpoS sigma subunits but others are transcribed by the RpoD

Abbreviations: AHL, \textit{N}-acylhomoserine lactone; AI, autoinducer; MCS, multiple-component system; PS-TF, promoter-specific transcription factor; QS, quorum sensing; TCS, two-component system; TF, transcription factor.

Two supplementary tables are available with the online version of this paper.
holoenzyme (Ballesteros et al., 1998). With respect to the regulation mode of ftsQAZ transcription, SdiA activates the upstream promoter through contact with the RpoD C-terminal region but inhibits the downstream promoter through binding competition with RNA polymerase (Yamamoto et al., 2001). Because of the lack of LuxI-type enzyme for AI-1 synthesis in E. coli, it is believed that SdiA senses AHLs produced by other bacteria in the environment (Garcia-Lara et al., 1996; Michael et al., 2001; Lee et al., 2007). If SdiA is a sensor of AHL-like signals present in the environment, it should control some stress-response genes besides the ftsQAZ operon. In fact, the involvement of the SdiA regulator has recently been reported for the expression of virulence, biofilm formation, adhesion, motility and multi-drug efflux genes (Ahmer et al., 1998; Wei et al., 2001; Rahmati et al., 2002; Lee et al., 2009; Dyszewski et al., 2010; Sharma et al., 2010; Tavio et al., 2010). The Genomic SELEX screening also indicated that a number of genes for cell differentiation and stress response are under the control of SdiA (T. Shimada and others, unpublished). If SdiA plays such a critical role in the control of cell differentiation and cell–cell communication, transcription of the sdiA gene should be under a complex regulation system, as identified for the global regulators such as csgD encoding the master regulator of biofilm formation (Ogasawara et al., 2010a, b, 2011), which is under the control of more than 20 transcription factors (TFs) (Ishihama, 2010, 2012). We then supposed that sdiA transcription should be under a complex regulation system involving a number of TFs. In contrast to the recent advance in understanding the functional role of SdiA, however, nothing is known about the regulation of expression of the sdiA gene.

To gain insight into the regulation of sdiA transcription, we tried in this study to identify the whole set of TFs involved in regulation of the sdiA promoter. As a short-cut approach for this purpose, we first set up an in vitro screening system of promoter-specific TFs (PS-TFs). Since most DNA-binding TFs in E. coli bind near promoters and interact with promoter-bound RNA polymerase for function (Ishihama, 2000, 2010, 2012), we searched for DNA-binding TFs that bind to a short DNA fragment of a test promoter simply by using PAGE. Using this PS-TF screening system, we analysed, in this study, a total of 191 TFs and identified at least 15 TFs with binding activity to the sdiA promoter, including five response regulators of the two-component system (TCS). The in vivo roles of these TCS TFs were analysed in detail with respect to regulation in vivo of the sdiA promoter.

METHODS

Bacterial strains and culture conditions. The complete genome sequence was determined for E. coli K-12 W3110 type-1 (Hayashi et al., 2006; Riley et al., 2006). E. coli W3110 type-1 (Jishage & Ishihama, 1997) was used for construction of the expression vectors of all TFs (Yamamoto et al., 2005; Shimada et al., 2005; Ogasawara et al., 2010b). E. coli BL21(DE3) [pLysS recA1 deoR hsdS thrD, thi hsdS2 thrD proAB lacIQZ M15 (supE44, deoR recA1 lacIQZ M15)] (Studier & Moffatt, 1986) was used for expression and purification of TFs. A set of E. coli K-12 BW25113 [lacY1 rpsL1 lacZΔM1515 trkA hsdR araBAD rhaBAD] mutants, each lacking the gene coding for one specific TF, were products of the Keio collection (Baba et al., 2006), and obtained from the National Bio-Resource Center (National Institute of Genetics, Japan). The strains used in this study are summarized in Table S1 (available in Microbiology Online). Cells were grown in LB at 37 °C with shaking and cell growth was monitored by measuring OD₆₀₀. Usually, overnight culture was diluted into fresh pre-warmed medium so as to make a constant cell density for wild-type and mutant cultures.

Plasmid construction. The expression plasmids of His-tagged forms of 300 species of E. coli TFs were constructed as described previously (Yamamoto et al., 2005; Shimada et al., 2005; Ogasawara et al., 2010b). In brief, a DNA fragment corresponding to the coding region of a test TF was amplified by PCR using E. coli K-12 W3110 type-1 genome DNA as a template and a pair of gene-specific primers. After digestion with EcoRI and BamHI, the PCR-amplified fragments were inserted into pRS551 (Simons et al., 1987) at the corresponding sites to generate the promoter assay vector pRS551-sdiA. The same sdiA promoter segment was used for construction of the sdiA–lux (luciferase) reporter plasmid pLux-sdiA using the pLux vector (Burton et al., 2010). Since pLux carries a Km resistance marker, the kam gene was removed from all the recipient strains using pCP20 (Cherepanov & Wackernagel, 1995).

Expression and purification of TFs. C-terminal His-tagged TFs were expressed in E. coli BL21(DE3) transformed with each of 200 expression plasmids and affinity-purified according to the standard purification procedure (Yamamoto et al., 2005; Shimada et al., 2005; Ogasawara et al., 2010b). In brief, the transformants were grown in LB media at 37 °C to an OD₆₀₀ of 0.6–0.7 and then IPTG was added to induce TF expression. The cells were harvested, suspended in a lysis buffer and disrupted by sonication. DNase-I was added into the cell lysate and incubated on ice for at least 3 h for digestion of associated genomic DNA fragments. The lysate was then centrifuged and the resulting supernatant solution was adjusted so as to contain a final concentration of 1 M NaCl and 20 mM imidazole. Samples were then adsorbed to a filter column of nickel-charged resin (Ni-NTA agarose; Qiagen). After washing the protein-bound column with a washing buffer (20 mM imidazole in a 50 mM phosphate, 500 mM NaCl buffer), the column-bound His-tagged TF was then eluted in an elution buffer (washing buffer containing 250 mM imidazole). The purity of each peak of the eluate was checked by SDS-PAGE. Peak fractions were pooled, dialysed by the storage buffer (20 mM Tris-HCl, pH 7.6, 5 mM magnesium acetate, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT and 50% glycerol) and stored at −80 °C prior to use. The purity of TFs used in this study ranged from 80 to 99% as checked by SDS-PAGE. For PS-TF screening for each TF, we used two or three different batches of different purity.

Gel-shift assay of SdiA-DNA complexes. Probes, carrying the 350 bp sdiA promoter or different 115 bp segments of this sdiA promoter, were generated by PCR amplification using a pair of primers (one 5′-FITC-labelled and another unlabelled), E. coli W3110 type-1 genome DNA (50 ng) as the template, and Ex Tag DNA polymerase. For the construction of the sdiA promoter–laclZ (β-galactosidase) reporter vector, a 450 bp sdiA promoter upstream from the initiation codon was prepared by PCR using E. coli K-12 W3110 type-1 genome DNA as a template and a pair of gene-specific primers. After digestion with EcoRI and BamHI, the PCR-amplified fragments were inserted into pRS551 (Simons et al., 1987) at the corresponding sites to generate the promoter assay vector pRS551-sdiA. The same sdiA promoter segment was used for construction of the sdiA–lux (luciferase) reporter plasmid pLux-sdiA using the pLux vector (Burton et al., 2010). Since pLux carries a Km resistance marker, the kam gene was removed from all the recipient strains using pCP20 (Cherepanov & Wackernagel, 1995).
polymerase (Takara). All the PCR products with FITC at 5' termini were purified by PAGE. Mixtures of sdiA promoter segments and TFs were subjected to gel-shift assay under the standard conditions (Ogasawara et al., 2007a, b).

**Measurement of sdiA promoter activity.** The test promoter—lacZ fusion plasmid pRS551-sdiA was transformed into *E. coli* AI-2073 (BW25113) and the otherwise isogenic mutant defectives lacking the TF gene (see Table 1). Transformants were grown in LB at 37 °C with shaking, and subjected to the β-galactosidase assay by adding o-nitrophenyl-D-galactopyranoside as described by Miller (1972). Cells for the luciferase assays were grown in LB in 96-well micro-plates, and the luciferase activity encoded by pLux-sdiA was measured following a standard procedure (Burton et al., 2010) using an automated plate reader.

**Northern blot analysis of sdiA mRNA.** Total RNAs were extracted from *E. coli* cells by the hot phenol method (Aiba et al., 1981). RNA purity was checked by electrophoresis on 0.8% agarose gel in the presence of formaldehyde followed by staining with methylene blue. The amounts of RNA used were measured by staining rRNAs with ethidium bromide. Northern blot analysis was performed essentially as described previously (Yamamoto & Ishihama, 2000; Maeda et al., 2007a, b). DIG-labelled probes were prepared by PCR amplification using *E. coli* strain AI-2053 (W3110) genomic DNA as template, DIG-11-dUTP (Roche) and dNTP as substrates, gene-specific forward and reverse primers, and Ex Taq DNA polymerase. Total RNAs were incubated in formaldehyde-MOPS gel-loading buffer for 10 min at 65 °C for denaturation, subjected to electrophoresis on formaldehyde-containing 1.5% agarose gel, and then transferred to nylon membrane (Roche). Hybridization was performed with the DIG easy Hyb system (Roche) at 50 °C overnight with a DIG-labelled probe. For detection of the DIG-labelled probe, the membrane was treated with anti-DIG-AP Fab fragments and with a DIG-labelled probe. For detection of the DIG-labelled probe, the membrane was treated with anti-DIG-AP Fab fragments and digoxigenin. The protein-blotted filter was treated with anti-TF antibodies, which were raised in rabbits against purified TFs. Antibodies were detected by chemiluminescence (Roche). The amounts of RNA used were measured by staining rRNAs with ethidium bromide. Northern blot analysis was performed essentially as described previously (Yamamoto & Ishihama, 2005). The sdiA probe was generated by PCR amplification with the primer pairs, sdiA-S1F (GCCAGTTTCTCGAGAAGTTTCTGCT) and 32P-labelled sdiA-S1R (GGTCTCATTCTCGTAAACGCCAAC). The labelled promoter fragment was incubated with 100 ng of total RNA in hybridization buffer (80% formamide, 0.4 M NaCl, 20 mM HEPES, pH 6.4) at 75 °C for 10 min, followed by incubation at 37 °C overnight for hybridization and then digested with S1 nuclease. The undigested materials were extracted with phenol, precipitated with ethanol, and analysed by electrophoresis on a polyacrylamide gel containing 6 M urea.

**Complementation of TFs.** Overexpression of TFs in mutants defective in TFs was performed using the ASKA library (Kitagawa et al., 2006), which was kindly provided by H. Mori (Nara Institute of Science and Technology). The reporter assay vector was then transformed for measurement of the sdiA promoter in the presence and absence of TF expression.

**Western blotting assay of TFs.** Intracellular concentrations of TFs were determined by using the quantitative Western blotting assay under standard conditions (Jishage & Ishihama, 1995; Jishage et al., 1996; Maeda et al., 2000; Ali Azam et al., 1999). In brief, *E. coli* whole lysates were prepared by sonication after lysozyme treatment, and directly subjected to SDS-PAGE. After transfer of proteins onto filters, the protein-blotted filter was treated with anti-TF antibodies, which were raised in rabbits against purified TFs. Antibodies were detected by chemiluminescence (Roche). The amounts of RNA used were measured by staining rRNAs with ethidium bromide. Northern blot analysis was performed essentially as described previously (Yamamoto & Ishihama, 2000). The sdiA probe was generated by PCR amplification with the primer pairs, sdiA-S1F (GCCAGTTTCTCGAGAAGTTTCTGCT) and 32P-labelled sdiA-S1R (GGTCTCATTCTCGTAAACGCCAAC). The labelled promoter fragment was incubated with 100 ng of total RNA in hybridization buffer (80% formamide, 0.4 M NaCl, 20 mM HEPES, pH 6.4) at 75 °C for 10 min, followed by incubation at 37 °C overnight for hybridization and then digested with S1 nuclease. The undigested materials were extracted with phenol, precipitated with ethanol, and analysed by electrophoresis on a polyacrylamide gel containing 6 M urea.

**RESULTS**

**Development of the PS-TF screening system**

A total of about 300 species of DNA-binding TFs exist in *E. coli* K-12 (Ishihama, 2010, 2012). Up to the present time, we have purified most of these regulators and used them for the identification in vitro of regulation targets of each TF by the improved Genomic SELEX screening (Shimada et al., 2011).

**Table 1. sdiA promoter-binding transcription factors**

<table>
<thead>
<tr>
<th>TF</th>
<th>Regulation phenotype</th>
<th>No. targets</th>
<th>Effector</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcrR</td>
<td>Multi-drug transport</td>
<td>3 (acrA, aecR, micF)</td>
<td>Proflavin</td>
</tr>
<tr>
<td>ArcA</td>
<td>Aerobic respiration</td>
<td>9 (ace, gfp, nuo)</td>
<td>ArcBA TCS</td>
</tr>
<tr>
<td>CpxR</td>
<td>Envelope stress</td>
<td>100 (csgD, ompF, rpoE)</td>
<td>CpxAR TCS</td>
</tr>
<tr>
<td>CRP</td>
<td>Carbon source utilization</td>
<td>350 (lacZ, araB, gac)</td>
<td>cAMP</td>
</tr>
<tr>
<td>Dan</td>
<td>Anaerobiosis adaptation</td>
<td>668 (tdtA; nucleoid protein)</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DeoR</td>
<td>Deoxynucleoside utilization</td>
<td>3 (tsx, nupG, deoC)</td>
<td>β-Glucoside</td>
</tr>
<tr>
<td>GusR</td>
<td>β-Glucoside utilization</td>
<td>2 (uidA, uidR)</td>
<td>β-Glucoside</td>
</tr>
<tr>
<td>MchR</td>
<td>Biofilm formation</td>
<td>3 (mchA, mchB, yciG)</td>
<td>QS AI-2</td>
</tr>
<tr>
<td>OmpR</td>
<td>Altered osmolarity</td>
<td>13 (ompA, ompF, csgD)</td>
<td>EnvZ-OmpR TCS</td>
</tr>
<tr>
<td>QseD</td>
<td>Flagella formation</td>
<td>5 (metN, cydA, fecA)</td>
<td>hypochloride</td>
</tr>
<tr>
<td>RcsB</td>
<td>Capsular synthesis</td>
<td>13 (gadA, hdeA, osmB)</td>
<td>RcsABFCD TCS</td>
</tr>
<tr>
<td>SdiA</td>
<td>Cell division control</td>
<td>20 (ftsQ, ydiV, gadW)</td>
<td>QS AI-1 (HSI)</td>
</tr>
<tr>
<td>TorR</td>
<td>TMAO respiration system</td>
<td>5 (torC, gadA, tnaA)</td>
<td>TorSR TCS</td>
</tr>
<tr>
<td>TpK</td>
<td>Cell division control</td>
<td>2 (rut, slmA)</td>
<td></td>
</tr>
</tbody>
</table>

The number of regulation targets are from RegulonDB and EcoCyc except for CRP, of which the targets have been identified by Genomic SELEX (Shimada et al., 2011a). Some representative promoters are described in parentheses.
et al., 2005). Some of the results have been published (reviewed by Ishihama, 2012). For most of the TFs used in this study, the collection of Genomic SELEX screening results included hitherto identified regulation targets. This indicates that most of the purified TFs are functional with respect to the recognition of target sequences. In this study, we used this collection of purified TFs for development of the in vitro screening system of PS-TFs, in which a DNA fragment of one test promoter is mixed with each of the purified TFs and then directly subjected to PAGE for detection of DNA–protein complexes. With the use of a mixture of specific and non-specific promoter fragments, the specificity of DNA binding can be easily identified in this PS-TF system.

Screening of sdiA PS-TFs

This PS-TF screening system was employed for detection of TFs involved in regulation of the sdiA gene. In the initial screening, we used a pair of fluorescently labelled DNA probes (0.5 pmol each), one 355 bp sdiA promoter segment (upstream from the initiation codon) and another 240 bp unrelated DNA segment from the yecC open reading frame as an internal reference. A total of 191 TFs (136 TFs with known function and 55 TFs with unknown function; hereafter referred to as known and unknown TFs, respectively) were affinity-purified as His-tagged forms (Table S2). Each TF (20 pmol) was mixed with the probe mixture in a volume of 10 μl and after incubation for 10 min at 37 °C, directly subjected to 5% PAGE. DNA-TF complexes migrated more slowly than the unbound probes and could be easily detected (see one example in Fig. 1a).

The initial screening was performed three times using three different preparations of purified TFs and a total of 15 TFs were selected as the candidate TFs involved in regulation of the sdiA promoter (Table 1). For some TFs, TF-sdiA promoter complexes did not form clear bands, forming smear bands, but the binding to the sdiA promoter could be detected based on the disappearance of unbound free probe. Even under the reaction conditions employed and at a fixed concentration of TF, some TFs could be identified as binding to the sdiA promoter segment but some others bound not only to the sdiA promoter but also to the unrelated yecC DNA fragment (for some typical examples see Fig. 1b).

Of these 15 sdiA promoter-specific TFs, five TCS regulators (ArcBA, CpxAR, EnvZ-OmpR, TorSR and RcsF/RcsC/ RcsD/RcsAB) were included (note that multiple phosphorelay system is involved in control of RcsAB). As expected, these five TCS TFs showed sdiA promoter-binding activity only in the presence of acetyl phosphate for phosphorylation in vitro of the respective response regulators. Besides these five TFs, ten other TF candidates (AcrR, CRP, Dan, DeoR, GruR, McbR, QseD, SdiA, Ttk and YnfL) are more or less involved in response to various stresses (Table 1). The sdiA promoter was indicated to be under the control of CRP, the best-characterized global TF, that regulates many as 350 target operons as identified by Genomic SELEX screening (Shimada et al., 2011a, 2013). CRP binds to the sdiA promoter only in the presence of cAMP as an obligatory effector that is formed in vivo in the absence of glucose for utilization of other carbohydrates.

Since the response to environmental stresses and the signal transduction pathways have been well characterized for the TCS regulators, we performed detailed analyses of the regulatory roles of the five TCS TFs in regulation of the sdiA promoter.

Confirmation of the sdiA PS-TFs: protein-dose-dependent complex formation

For confirmation of the binding specificity of the five TFs to the sdiA promoter, we next analysed the protein-dose-dependent formation of sdiA promoter-TF complexes. All five TCS TFs formed sdiA promoter complexes in a protein-dose-dependent manner (Fig. 2). In this second series of PS-TF assays, we could identify the strength of sdiA promoter-binding affinity for each TF. If the level of active protein is the same between these five TFs, the affinity order was estimated to be: TorR>ArcA>RcsB>CpxR>OmpR, as judged from the minimum amount of protein needed to form sdiA promoter complexes (Fig. 2). Under the TF concentrations analysed, the sdiA promoter segment added was all converted into TF complexes for four TFs, CpxR, ArcA, TorR and RcsB, but not for OmpR, which was partially converted to sdiA promoter-OmpR complexes under the protein concentrations used (Fig. 2). Upon further increase in OmpR addition, however, the sdiA promoter segment was completely converted to OmpR complexes (data not shown).

Mapping of the binding sites of TFs on the sdiA promoter

Next we tried mapping of the binding site of each sdiA promoter-specific TF on the sdiA promoter. For this purpose, we constructed a set of four DNA fragments of 115 bp in length, starting from the 350 bp sdiA promoter sequence upstream from the initiation codon and each overlapping by 30 bp sequence (Fig. 3). Protein-dose-dependent formation of TF-DNA complexes was examined for each of the sdiA promoter segments. Four transcription initiation sites have been predicted for the sdiA promoter between −25 and −85 from the initiation codon (Yakhnin et al., 2011).

Binding of OmpR and TorR was identified for two sdiA promoter-proximal segments, sdiA-c and sdiA-d (Fig. 3), indicating that OmpR and TorR bind to different sites located in each of two probes or a single site (−85 to −115 bp) that overlaps these two probes. The binding of AcrR and CpxR was identified for all three sdiA promoter segments, sdiA-b, sdiA-c and sdiA-d, while RcsB bound to all four sdiA promoter segments. This indicates that AcrR,
PS-TF screening for the sdiA promoter

Fig. 1. PS-TF screening of the sdiA promoter. (a) The PS-TF screening was performed for a search of TFs involved in regulation of the sdiA promoter. A mixture of 0.5 pmol each of a 355 bp fluorescently labelled sdiA promoter and a 240 bp fluorescently labelled yecC DNA probe, added as an internal unrelated reference, was incubated with 20 pmol each of a total of 191 purified TFs in a total volume of 10 µl, and directly subjected to PAGE analysis. This PAGE pattern shows the gel-shift assay for TFs with known functions. Filled triangles indicate the sdiA promoter probe while open triangles indicate the unrelated yecC ORF probe. Stars indicate TF candidates with specific binding to the sdiA promoter in this assay. After three independent PS-TF screenings using three different TF preparations, a total of 15 TFs were identified, which specifically bind to the sdiA probe but not to unrelated yecC ORF probe. (b) A PAGE pattern of some representative TFs without DNA-binding activity, and with the binding activity to the sdiA promoter or both the sdiA promoter and unrelated yecC ORF probe. In the reactions of EnvR, BaeR and ArcA, acetyl phosphate was added for TF phosphorylation in vitro. Small triangles along each gel lane indicate TF-DNA probe complexes.

Fig. 2. Protein-dose-dependent formation of sdiA promoter-TF complexes. A mixture of 0.5 pmol each of fluorescently labelled sdiA promoter and unrelated yecC DNA was incubated with increasing concentrations of TCS and MCS TFs, and directly subjected to PAGE analysis. The TF concentrations used were (from lane 1 to lane 5): CpxR and ArcA, 0, 2.5, 5.0, 7.5 and 10 pmol; OmpR, 0, 2.5, 5.0, 10 and 15 pmol; TorR, 0, 5, 10, 15 and 20 pmol; and RcsB, 0, 2.5, 5.0 pmol. The slow-migrating band represents the sdiA promoter probe while the fast-migrating band represents the unrelated yecC ORF probe added as an internal reference.
CpxR and RcsB are capable of binding more than one site along the sdiA promoter region.

**Regulation in vivo of the sdiA promoter: reporter assays**

Five TCS TFs have been indicated to specifically interact in vitro with the sdiA promoter. Next we examined possible influence of these five TFs on regulation in vivo of the sdiA promoter. First we employed the reporter assay using lacZ coding for β-galactosidase. A multicopy plasmid carrying the sdiA promoter–lacZ open reading frame fusion was transformed into wild-type E. coli and mutant strains, each lacking arcA, cpxR, ompR, rcsB or torR. Transformants were grown in LB at 37 °C and measured for β-galactosidase activity at various times. The level of lacZ expression in mutant transformants with deletions in the genes for all five TFs was higher than that in wild-type transformants (Fig. 4a), implying that all these TFs are repressors for the sdiA promoter. In particular, the lacZ activity in the ompR mutant was more than threefold higher than the wild-type.

The β-galactosidase activity in wild-type cells was high at the exponential phase of growth and then gradually decreased upon transfer to the stationary phase, but the LacZ activity in the ompR mutant was significantly higher than that in the wild-type throughout the growth phases from 4 to 12 h (Fig. 4a). For detection of LacZ reporter activity, we used a multicopy plasmid vector. In this case, high levels of TFs should be needed for effective control of the sdiA promoter. We then employed a single-copy vector for the reporter assay.

To confirm the regulatory roles of test TFs, we next performed a more sensitive reporter assay using a single-copy vector of the bacterial luciferase, which catalyses a bioluminescent oxidation of reduced flavin mononucleotides (Close et al., 2009). The sdiA promoter–lux reporter vector was transformed into wild-type E. coli and mutants, each lacking one test TF gene except for the arcA-defective mutant, into which the sdiA–lux plasmid could not be transformed. Transformants were grown in LB at 37 °C using a microplate and the fluorescent emission was monitored at various times. In this case, the luciferase (Lux) activity in wild-type E. coli was maximal at 8 h of the microplate culture. The difference in the time for maximum activity in the wild-type culture between two reporter assays is attributable to the difference in growth rate, i.e. liquid tube culture for LacZ assay and microplate culture for Lux assay. The growth of Lux assay culture was about 1.5-fold slower than the LacZ assay culture.

In agreement with the LacZ reporter assay, the luciferase activity increased for all the mutants (Fig. 4b), supporting the prediction that all the test TCS TFs are repressors of the sdiA promoter. The enhancement of reporter activity was high for the ompR mutant in both exponential phase (4 h) and late-exponential phase (8 h). Besides the ompR mutant, the rcsB mutant showed more than threefold increase in Lux activity than the wild-type for both 4 and 8 h culture. The successful detection of RcsB effect was attributable to the increased sensitivity in the reporter assay with use of a single-copy vector.

In order to confirm that the increase in sdiA promoter-directed reporter activity in the TF mutant strains was...
attributable to the lack of respective TFs, we next examined the influence of overexpression of two model TCS TFs, OmpR and RcsB, which exhibited marked repression of the sdiA promoter. IPTG-inducible expression plasmids of OmpR and RcsB were transformed to both wild-type E. coli and the respective mutants, and the Lux activity was examined (Fig. 4c). In the presence of overexpression in trans of OmpR and RcsB, the Lux activity decreased for both wild-type and mutant strains, and the Lux level became essentially the same between the wild-type and TF-defective mutant (Fig. 4c). These results further support the repression role for OmpR and RcsB.

**Intracellular levels of five TCS-MCS TFs**

The regulation levels of test TFs on the sdiA promoter should be influenced by the intracellular levels of these TFs. We then tried to measure the intracellular concentrations of all five test TCS TFs. For this purpose, we raised anti-TF antibodies in rabbits by injecting the purified TFs. Under
the same culture conditions employed for the reporter assay, we measured the intracellular concentrations of TFs using quantitative Western blot analysis (Jishage & Ishihama, 1995; Jishage et al., 1996; Maeda et al., 2000; Ali Azam et al., 2000). The intracellular level of RpoA stayed constant throughout the growth phase (Ishihama, 2000); the level of each TF was quantified as a relative value to the RpoA level. The TF level was the highest for OmpR, ranging from 300 molecules per genome at exponential growth phase to 1200 molecules per genome in stationary phase (Table 2). The order of intracellular concentration in growing E. coli cells was: OmpR>RcsB>CpxR>ArcA>TorR. Noteworthy is that this order correlates with the repression order of the sdiA promoter (see Fig. 4). For all five TFs, the intracellular level increased in the stationary phase of cell growth, implying that the level of these TFs increases concomitant with the transition into stressful stationary phase in agreement with their physiological roles (see Discussion).

Regulation in vivo of the sdiA promoter: mRNA assays

Finally the level of sdiA mRNA was directly measured for the ompR and rcsB mutants, which showed marked increases in sdiA promoter activity by both LacZ and Lux reporter assays, and regained the repression activity by expression in trans of OmpR or RcsB proteins (see Fig. 4). Total RNA was isolated and subjected to Northern blot analysis using the sdiA-specific probe. In the ompR mutant, marked increase of sdiA mRNA was observed (Fig. 5a). The level of sdiA mRNA also increased significantly in the rcsB mutant albeit at a lower level (Fig. 5a). Again, these findings support the repression role of OmpR and RcsB in the sdiA promoter.

A set of TCS TFs was found to be involved in regulation of the sdiA promoter. This finding raises a possibility that the sdiA promoter should be influenced by changes in culture conditions. The role of OmpR is well characterized in response to external conditions. We then examined some of the well-characterized stressful culture conditions. Marked induction of sdiA mRNA was observed by exposure to high salt by adding 2 % NaCl (Fig. 5b), indicating that the activated form of OmpR is responsible for repression of the sdiA promoter. We then propose that, when the TCS response regulators are activated under stressful conditions, the sdiA promoter is repressed, ultimately leading to inhibition of the ftsQAZ operon encoding the cell division apparatus.

The intracellular level of TCS TFs in growing cells is low for ArcA (150 molecules per genome) and TorR (100 molecules per genome) (see Table 2). Accordingly, the detection of influence of ArcA and TorR on the sdiA promoter was low as measured by the reporter assays (see Fig. 4) and the Northern blot analysis (data not shown). We then analysed sdiA mRNA using an S1 nuclease assay and in the presence and absence of overexpression of ArcA and TorR (Fig. 5c). In the S1 nuclease assay, we detected sdiA transcription from P1, P2 and P3 promoters. Expression of both ArcA and TorR was found to repress sdiA transcription as well as OmpR, supporting the prediction that ArcA and TorR are also the repressors of the sdiA promoter as in the cases of OmpR, RcsB and

Table 2. Intracellular concentrations of TCS TFs

<table>
<thead>
<tr>
<th>TF</th>
<th>Exponential phase</th>
<th>Stationary phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ArcA</td>
<td>&lt;100</td>
<td>850</td>
</tr>
<tr>
<td>CpxR</td>
<td>150</td>
<td>1000</td>
</tr>
<tr>
<td>OmpR</td>
<td>300</td>
<td>1200</td>
</tr>
<tr>
<td>RcsB</td>
<td>250</td>
<td>1300</td>
</tr>
<tr>
<td>TorR</td>
<td>&lt;100</td>
<td>450</td>
</tr>
<tr>
<td>RpoA</td>
<td>5000</td>
<td>5000</td>
</tr>
</tbody>
</table>

*E. coli* K-12 W110 type-1 was grown in LB at 37 °C. Cells were harvested at both exponential growth and stationary phases. Whole-cell lysate was subjected to quantitative Western blot analysis using specific antibodies against each TF. The concentration was calculated on the basis of intracellular level of RNA polymerase subunit, that is 5000 molecules per genome equivalent of DNA (Ishihama, 2000). The values represent the means of two independent determinations using two independent cultures.

Fig. 5. Measurement of sdiA mRNA. (a) Northern blot analysis. Total RNA was extracted from wild-type and ompR mutants. The level of sdiA mRNA was measured by Northern blot analysis while the total amounts of RNA analysed were measured by staining rRNAs with ethidium bromide. (b) Northern blot analysis under various growth conditions. Wild-type *E. coli* culture was exposed to the indicated stress: high salt by adding 2 % NaCl; alkaline pH 9.0; and anaerobic culture in an anaerobic chamber. (c) S1 nuclease analysis. Total RNA was extracted from wild-type and mutants, each containing a member of a pBAD series of arabinose-inducible TF expression vectors. pBAD33, vector; pBADtorR, TorR expression plasmid; pBADarcA, ArcA expression plasmid; and pBADompR, OmpR expression plasmid. The level of sdiA mRNA was measured by S1 nuclease assay. The site of transcription initiation, P1, P2 and P3, was determined by the Maxam-Gilbert sequence ladder that was run in parallel with the S1 nuclease samples.
SdiA was identified as a regulator of the ftsQAZ operon. It should be noted, however, that the culture conditions (LB medium under aeration) employed in this study do not represent the optimal conditions for detection of the effect of TCS regulators examined in this study.

DISCUSSION

PS-TF screening system

A Genomic SELEX screening system has been developed for identification of the whole set of regulation targets of DNA-binding TFs (Shimada et al., 2005; Ishihama, 2010, 2012). So far, we have identified the regulation targets for more than 200 TFs from *E. coli* (reviewed by Ishihama, 2012). Results indicated that the number of regulation targets of *E. coli* TFs is much more than the known targets listed in RegulonDB (Salgado et al., 2013) and EcoCyc (Keseler et al., 2013), which were mostly identified by ordinary molecular genetic studies of individual TFs or predicted using the known TF-binding consensus sequences. For instance, the number of regulation targets of the best-characterized CRP (cAMP receptor protein or catabolite activator protein) increased to approximately 350 from the 150 known targets (Shimada et al., 2011a). The accumulation of regulation target data for more than 200 TFs obtained by Genomic SELEX screening allows the identification of a group of TFs involved in regulation of a specific promoter. For instance, more than 20 TFs have been identified as being involved in regulation of the cgD gene encoding the master regulator of biofilm formation (Ogasawara et al., 2010a, b, 2011; Ishihama, 2012). Identification of the whole set of TFs involved with one specific promoter, however, awaits the identification of regulation targets for all 300 TFs.

For a short-cut approach in the search for TFs involved in regulation of one specific promoter, we have developed in this study a PS-TF screening system, in which mixtures of a specific promoter and an unrelated DNA were incubated with various TFs for identification of promoter-binding TFs by gel-shift assay. TFs with binding affinity only to the test promoter can be easily identified by PAGE as forming promoter-TF complexes. Here we showed a successful application of the PS-TF system, in which a pair of sdiA promoter and unrelated yecC DNA segments was used for a search of TFs that bind only to the sdiA promoter probe. As an extension, the PS-TF system can be applied for simultaneous search of TFs against multiple promoters in a single reaction. For this purpose, the test promoter probes are designed to be different in size so as to allow clear separation of all promoter probes on a single PAGE gel. Using this mixed gel-shift assay, we could easily identify a set of TFs for each of the test promoters.

The list of regulation targets of the SdiA TF identified by Genomic SELEX screening, however, includes a number of stress response genes and genes expressed in stationary phase (T. Shimada and others, unpublished). In good agreement with this finding, we have identified, in this study, the involvement of at least 15 TFs in the regulation of the sdiA promoter using the newly developed PS-TF screening system. Most of these TFs are involved in regulation of stress-response genes and genes that are expressed in stationary phase. Here we focused detailed analysis on five TCS response regulators, ArcA, CpxR, TorR, OmpR and RcsB.

Among the five TCS TFs, the repression of the sdiA promoter is more significant for both RcsB and OmpR (see Figs 4 and 5) supposedly because the intracellular levels of these two TFs under the steady-state culture conditions herein employed are higher than those of the other three TFs (see Table 2). The possibility is, however, not ruled out that this difference in the repression level is due to the different level of TF activation. The derepression of the sdiA promoter is most significant for the ompR mutant. The EnvZ-OmpR TCS senses changes in extracellular osmolarity, and the phosphorylated OmpR regulates transcription of several operons whose products are involved in adaptation to changes in osmolarity by selective expression of outer membrane porins (Egger et al., 1997; Yoshida et al., 2006). In addition, OmpR has been recognized to be involved in control of many stress responses, including biofilm formation (Prigent-Combaret et al., 2001; Ogasawara et al., 2010b), curli fimbriae formation (Jubelin et al., 2005), drug export (Hirakawa et al., 2003), peptide transport (Goh et al., 2004) and cell morphology (Yamamoto et al., 2000). The finding of sdiA regulation by OmpR highlights the global role of OmpR in functional and structural modulation of *E. coli* upon exposure to high osmolarity. RcsB is a response regulator that belongs to the multi-component RcsF/RcsC/RcsD/RcsA-RcsB phosphorelay system, and forms complexes with the RcsA auxiliary protein as a heterodimer (Majdalani & Gottesman, 2005). In addition to the cognate sensor kinase RcsC, RcsF is able to phosphorylate RcsB, forming an MCS signal transduction pathway (Gupte et al., 1997).

The Rcs system senses extracellular changes in temperature, osmolarity and membrane distortion (Mouslim et al., 2003). RcsB has been recognized as a positive regulator of the cell division gene fisZ (Carballes et al., 1999). Here, we demonstrated that RcsB is a negative regulator of the sdiA gene, which in turn controls positively the ftsQAZ operon. Thus, the Rcs regulation system is involved in control of both upstream SdiA regulator and its target ftsQAZ operon.

Under anaerobic growth conditions, ArcA, the response regulator of the ArcBA TCS for anaerobic respiratory control (Iuchi & Weiner, 1996; Gunsalus & Park, 1994), represses the operons for respiratory metabolism such as the TCA cycle and glyoxylate shunt. Upon exposure to multiple stimuli leading to membrane disorder such as heavy metals, alkaline pH and high osmolarity, CpxR, the response regulator of the CpxAR TCS, regulates a set of...
genes in repair of membrane-associated proteins (Danese & Silhavy, 1997; Pogliano et al., 1997; Raivio et al., 1999). CpxAR was found to be involved in control of cell division as well as in control of motility, chemotaxis and biofilm formation (Jubelin et al., 2005; Dorel et al., 2006). Here, we examined the possible influence of CpxR in the absence of external stresses (see Figs 4 and 5). CpxR is activated directly by acetyl phosphate, a metabolite of the PTA-ACK pathway, in the absence of CpxA action (Wolfe et al., 2008). TorSR TCS, originally identified as a positive regulator for the genes related to trimethylamine N-oxide (TMAO) (Pascal et al., 1991), is involved in the defence against alkaline and acid stresses (Bordi et al., 2003) and in metabolic control under anaerobic conditions (García-Horsman et al., 1994).

CONCLUSIONS

The PSI-TF screening system was successfully employed for detection of TFs involved in regulation of the promoter for the E. coli sdiA gene encoding the master regulator of cell division and QS. After screening of 191 purified E. coli TFs, at least 15 TFs have been identified to bind the sdiA promoter, including five TCS regulators, ArcA, CpxR, OmpR, RcsB and TorR. After detailed functional analysis of these five TFs, all these TFs were found to be involved in repression of the sdiA promoter provided that the TFs are expressed and activated under specific stress conditions. Analysis of the regulatory roles of ten other TF candidates with binding activity to the sdiA promoter is in progress.

ACKNOWLEDGEMENTS

This work was supported by Grant-in-Aid for Grants-in-Aid for Scientific Research (A) (21241047), (B) (18310133) and (C) (25430173) to A.I., and MEXT-Supported Program for the Strategic Research Foundation at Private Universities 208-2012 (S0801037). We also thank Hitoriota Mori (Nara Institute of Science and Technology) for the gift of the ASKA library and Peter Lund (University of Birmingham) for the gift of pLux.

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Edited by: D. Grainger