Transcription factor CecR (YbiH) regulates a set of genes affecting the sensitivity of *Escherichia coli* against cefoperazone and chloramphenicol

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Genomic SELEX (systematic evolution of ligands by exponential enrichment) screening was performed for identification of the binding site of YbiH, an as yet uncharacterized TetR-family transcription factor, on the *Escherichia coli* genome. YbiH was found to be a unique single-target regulator that binds *in vitro* within the intergenic spacer located between the divergently transcribed *ybiH-ybhGFSR* and *rhlE* operons. YbhG is an inner membrane protein and YbhFSR forms a membrane-associated ATP-binding cassette (ABC) transporter while RhlE is a ribosome-associated RNA helicase. Gel shift assay and DNase footprinting analyses indicated one clear binding site of YbiH, including a complete palindromic sequence of AATTAGTT—AACTAATT. An *in vivo* reporter assay indicated repression of the *ybiH* operon and activation of the *rhlE* operon by YbiH. After phenotype microarray screening, YbiH was indicated to confer resistance to chloramphenicol and cefazolin (a first-generation cephalosporin). A systematic survey of the participation of each of the predicted YbiH-regulated genes in the antibiotic sensitivity indicated involvement of the YbhFSR ABC-type transporter in the sensitivity to cefoperazone (a third-generation cephalosporin) and of the membrane protein YbhG in the control of sensitivity to chloramphenicol. Taken together with the growth test in the presence of these two antibiotics and *in vitro* transcription assay, it was concluded that the hitherto uncharacterized YbiH regulates transcription of both the bidirectional transcription units, the *ybiH-ybhGFSR* operon and the *rhlE* gene, which altogether are involved in the control of sensitivity to cefoperazone and chloramphenicol. We thus propose to rename YbiH as CecR (regulator of cefoperazone and chloramphenicol sensitivity).

INTRODUCTION

*Escherichia coli* contains a total of approximately 4500 protein-coding sequences on its genome (Blattner *et al.*, 1997; Hayashi *et al.*, 2006). The selection of genes for expression and their expression level are determined by controlling the utilization of a limited number of transcription apparatuses on the genome (Ishihama, 2000, 2012). A total of seven species of sigma factor, the promoter recognition subunit of RNA polymerase (RNAP) and about 300 species of transcription factor (TF) are together involved in this gene selection process (Riley *et al.*, 2006; Ishihama, 2009, 2010). At present, however, the regulatory functions are not known for about one-fifth of the *E. coli* TFs (Ishihama *et al.*, 2016). In the absence of knowledge of factors and conditions affecting the expression of regulatory functions, the ordinary genetic approach is not useful for identification of regulator targets of hitherto uncharacterized TFs. Bacterial DNA-binding TFs bind to DNA and regulate the genes nearby, and thus the prediction of regulation target promoters, genes and operons is possible based on knowledge of the location of recognition sequences by test TFs. As a short cut for identification of the regulation targets by uncharacterized TFs, we have developed an improved

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**Abbreviations:** ABC, ATP-binding cassette; CecR, regulator of cefoperazone and chloramphenicol sensitivity; MFS, major facilitator superfamily; NTA, nitrilotriacetic acid; PG, peptidoglycan; PM, phenotype microarray; RNAP, RNA polymerase; RND, resistance nodulation cell division; SELEX, systematic evolution of ligands by exponential enrichment; TF, transcription factor.
system of genomic SELEX (systematic evolution of ligands by exponential enrichment) (Shimada et al., 2005), and successfully applied it for identification of the regulation targets for a number of TFs (see Ishihama, 2012; Ishihama et al., 2016). The genomic SELEX screening system is particularly useful for identification of regulation targets of hitherto uncharacterized TFs. For instance, we have identified the regulation targets and regulatory roles for YeaM (renamed as RhlE), YbdC (renamed as RhlR), YdcN (renamed as SutR), YdhM (renamed as NemR) and Ygp (renamed as Dan) (Ishihama et al., 2016).

CecR (renamed YbiH) has been recognized as one of the uncharacterized TFs in E. coli and belongs to the TetR family (Ramos et al., 2005; Ishihama, 2012). A total of 15 member TFs are classified within the TetR family (Ishihama et al., 2016). In this study, a systematic search was performed for identification of the target genes under the control of CecR (regulator of cefoperazone and chloramphenicol sensitivity) by using the genomic SELEX system (Shimada et al., 2005). As a result, one strong CecR-binding site was identified only at a single site located within the intragenic spacer of the divergently transcribed ybhGFSR and rhlE operons. The cecR gene is located at the promoter-proximal end of the leftward five-member operon (on the linear map of the E. coli K-12 genome) while the rightward rhlE gene forms a single-gene operon (Fig. 1). Downstream of the cecR gene, the ybhG gene encoding a predicted inner memory protein and the ybhFSR genes encoding an ATP-binding cassette (ABC)-type transporter with an as yet unidentified function exist in this order. By contrast, the rightward operon encodes RhlE, one of six known RNA helicases in E. coli, but its physiological role remains unidentified. The purified CecR protein alone was found to bind to a palindromic sequence within the intergenic spacer between the cecR and rhlE operons. As an attempt to identify the regulatory function of uncharacterized CecR, we examined the characteristics of an E. coli strain lacking cecR by using the phenotype microarray system (Bochner et al., 2001; Ogasawara et al., 2015). The cecR mutant was found to be sensitive to several antibiotics. After checking the sensitivity to each antibiotic in liquid cultures, we focused on two antibiotics, cefoperazone and chloramphenicol, for detailed analyses. To identify which of the regulatory target genes of CecR is involved in the antibiotic sensitivity, we then checked the sensitivity to two antibiotics of E. coli mutants, each lacking one of the CecR-target genes. The results indicated that YbhFSR ABC-transporter is involved in the sensitivity control to cephalosporin while YbhG membrane protein is involved in the sensitivity control to chloramphenicol. Reporter assay indicated negative control of the cecR-ybhFSR operon by CecR. By contrast, RhlE helicase was suggested to be under the positive control of CecR. Taking the results together we propose that CecR (renamed YbiH) is a bifunctional regulator, repressing the cecR-ybhFSR operon and activating the rhlE operon, all being involved in the sensitivity control to two antibiotics, cefoperazone and chloramphenicol.

**METHODS**

**Bacterial strains.** The E. coli K-12 W3110 type-A (Ishage & Ishihama, 1997) was used to make the genome DNA library for the genomic SELEX screening of CecR-binding sequences, and for cloning of both the cecR gene and the CecR-regulated promoters. E. coli DH5α (F′ lacZΔM15 Δ proAB rpsL::Tn10 [Tn5 KmR]) was used for the construction of plasmids. E. coli BL21(DE3) (F ompT hsdS B(λDE3) gal dcm lacI(λ)) was used for propagation of the cloning vectors. E. coli BW25113, and its cecR mutant JW1788, ybhG mutant JW0779, ybhF mutant JW5104 and rhlE mutant JW0781 in the Keio collection (Baba et al., 2006) were obtained from the E. coli stock centre of the National Bio-Resource Project and used for characterization of the respective mutants with respect to antibiotic sensitivity.

Cells were cultured in Luria-Bertani (LB) medium or M9-0.4% glucose medium at 37°C. When necessary, ampicillin was added to the medium at a final concentration of 0.1 mg ml⁻¹. Cell growth was monitored by measuring the turbidity at 600 nm.

**Preparation of RNAP RpoD holoenzyme.** RNAP was purified from E. coli K-12 W3350 by using standard procedures (Fujita & Ishihama, 1996). The purified RNAP in storage buffer containing 50% glycerol was dialysed against the same buffer but containing 5% glycerol (v/v), and fractionated by phosphocellulose column chromatography in the presence of 5% glycerol to purify the sigma-free core enzyme. RNAP RpoD holoenzyme was reconstituted by mixing the purified core enzyme with a two-fold molar excess of purified RpoD sigma subunit (Fujita & Ishihama, 1996; Shimada et al., 2014).

**Expression and purification of His-tagged CecR protein.** Expression and purification of His-tagged CecR was performed according to standard procedures (Yamamoto et al., 2005; Ishihama et al., 2014). For construction of plasmid for CecR expression, a DNA fragment corresponding to the CecR-coding sequence was amplified by PCR and cloned into pET21a (+) (Novagen) between NdeI and NotI sites, leading to construct pCecR. For protein expression, pCecR plasmid was transformed into E. coli BL21(DE3). Transformants were grown in LB medium and the expression of CecR was induced by adding 1 mM IPTG at the mid-point of the exponential growth phase.

CecR protein was purified by the affinity purification procedure with use of a Ni-nitrilotriacetic acid (Ni-NTA) agarose column essentially according to standard procedures (Yamamoto et al., 2005; Ishihama et al., 2014). The affinity purified CecR protein was stored frozen in the storage buffer (50 mM Tris-HCl, pH 7.6 at 4°C, 200 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT and 50% glycerol) at −80°C until use. Protein purity was more than 95% as checked by SDS-PAGE.

**SELEX search for CecR-binding sequences.** The genomic SELEX system was used as described previously (Shimada et al., 2005, 2011a, b). The mixture of E. coli genome segments of 150–300 bp in length was prepared by PCR amplification using the E. coli DNA library (Shimada et al., 2005). For the genomic SELEX screening of CecR-binding sequences, 5 pmol of DNA fragments and 10 pmol of His-tagged CecR were mixed in a binding buffer (10 mM Tris-HCl, pH 7.8 at 4°C, 3 mM Mg acetate, 150 mM NaCl and 1.25 μg ml⁻¹ BSA) and incubated for 30 min at 37°C. The mixture was applied onto a Ni-NTA column, and after washing unbound DNA with the binding buffer containing 10 mM imidazole, DNA-CecR complexes were eluted with an elution buffer containing 200 mM imidazole. This SELEX cycle was repeated three times for enrichment of CecR-binding sequences. DNA
was isolated from DNA-CecR complexes by PAGE and amplified by PCR.

Mapping of SELEX fragments along the *E. coli* genome was also performed by SELEX-clos (cloning-sequencing) and SELEX-chip systems (Teramoto et al., 2010; Shimada et al., 2014, 2011b). For SELEX-chip analysis, a 43 450-feature DNA microarray (Oxford Gene Technology) was used under standard procedures (Shimada et al., 2005). The genomic SELEX sample obtained with use of CecR was labelled with Cy5, while another SELEX sample obtained in the absence of CecR addition was labelled with Cy5. After hybridization of samples to the DNA tiling array, the Cy5/Cy3 ratio was measured and the peaks of scanned patterns were plotted against the positions of DNA probes along the *E. coli* K-12 genome.

**RESULTS**

**Screening of CecR-binding sequences by genomic SELEX**

For the identification of DNA sequences that are recognized by *E. coli* CecR, we employed the genomic SELEX screening system (Shimada et al., 2005) using a complete library of *E. coli* genome DNA fragments instead of synthetic oligonucleotides with all possible sequences that were used in the original SELEX method (Ellington & Szostak, 1990; Tuerk & Gold, 1990). This library contains a mixture of 150–300 bp DNA fragments of the *E. coli* genome, altogether covering approximately 6.5-times the genome length. For SELEX screening, an aliquot of this DNA library was mixed with a two-fold molar excess of the purified His-tagged CecR protein, and the CecR-DNA complexes were affinity-purified using nickel-charged NTA agarose. In the early stage of this genomic SELEX cycle, the CecR-bound DNA fragments gave smear bands on PAGE as did the original genome fragment mixture. In the case of CecR, several discrete bands were identified under smear background after three SELEX cycles (data not shown), indicating that some DNA fragments with high affinity to CecR were enriched.

**Identification of regulation targets by CecR: genomic SELEX screening**

To identify the whole set of binding sequences of CecR, we then subjected the genome of *E. coli* to the cloning–sequencing system (SELEX-clos) and the DNA tiling array analysis (SELEX-chip) (Teramoto et al., 2010; Shimada et al., 2011a, b). In the SELEX-clos system, more than 90% of the independently isolated clones contained DNA fragments from the 228 bp intergenic spacer between the *secR* and *rhlE* genes (data not shown), indicating a high-level affinity of CecR to this region. Detailed mapping was performed using the SELEX-chip method. Genomic SELEX fragments were labelled with Cy5 while the original DNA library was labelled with Cy3. The mixture of fluorescently labelled samples was hybridized to a DNA tiling microarray (Oxford Gene Technology) (Teramoto et al., 2010; Shimada et al., 2011a, b). For elimination of bias in the library DNA, the ratio of fluorescence intensity bound to each probe between the test sample and the original library DNA was measured, and plotted against the corresponding position on the *E. coli* genome (Fig. 1). On the DNA tiling array used, a total of 43 450 probes, each consisting of a 60mer nucleotide, are aligned along the *E. coli* genome at 105 bp intervals, and therefore approximately 150–300 bp SELEX fragments should bind to two or more instructions (Biolog) (Bochner et al., 2001; Ogasawara et al., 2015). In this study, PM was used for screening of the phenotypic differences between the wild-type strain and the *cecR* mutant strains. Growth differences were monitored by measuring the colour intensity of oxidation of tetrazolium violet by NADH.
consecutive probes. This criterion was employed for identification of positive peaks of CecR binding. It was remarkable that only a single peak was identified in the SELEX-chip pattern (Fig. 1), in the intergenic spacer between cecR and rhlE. This SELEX-chip pattern agrees well with the isolation of a large number (more than 90%) of SELEX-clos clones containing sequences from the same region.

After SELEX-chip analysis, we realized that CecR is a unique and rare TF which binds in vitro to a single site on the E. coli K-12 genome between the divergently transcribed cecR and rhlE operons. This finding immediately indicates that CecR regulates either one or both of these flanking operons (see Fig. 1). The cecR operon includes, beside the cecR gene itself, the ybhG gene encoding an inner membrane protein with unknown function and the ybhFSR genes encoding an ABC-type transporter with unknown function in this order. By contrast, the rhlE gene forms a single-gene operon.

Identification of CecR-binding DNA sequences

To confirm the binding of CecR protein to the cecR-rhlE spacer and to identify its binding sequence, we performed gel-shift and DNase-I protection assays. By the gel-shift assay, the cecR-rhlE spacer segment of 228 bp in length formed a clear single band of CecR–probe complex in a protein-dose-dependent manner (Fig. 3b), indicating the binding of a single protomer of CecR to this spacer region. Under the same conditions, CecR did not bind to a 311 bp reference probe corresponding to the N-terminal sequence of the cecR ORF (Fig. 3a). As the TetR-family TFs form dimers under physiological conditions, the cecR protomer must be a dimeric form (Ramos et al., 2005).

For identification of the recognition sequence of CecR, we next carried out a DNase-I footprinting assay using the same 228 bp cecR-rhlE spacer probe. After forming DNA–CecR complexes in the presence of a fixed amount of the fluorescently labelled DNA probe and increasing concentrations of CecR, DNase-I treatment was carried out for a short period, and the partially digested DNA products were analysed by PAGE (Fig. 4a, lane CecR alone). A clear protection band of 27 bp in length was identified (Fig. 4b, bold underline), which includes a 20 bp inverted repeat sequence (AATTAGTTTACTAATTT) (Fig. 4b, bold letters), hereafter referred to as the CecR box. Although there should be two promoters for bidirectional transcription of the ybh and rhlE operons (Fig. 4c), RNAP RpoD holoenzyme alone protected a single 79 bp sequence (Fig. 4a, lane RNAP alone; and Fig. 4b, dotted underline). The RNAP RpoD holoenzyme alone recognizes and binds to the constitutive promoter with the typical TTGACA-(17 bp)-TATAAT sequence (Shimada et al., 2014). Within the region protected by RNAP, a constitutive promoter-like sequence, TTGAgg—TAgAAT, exists at −57 upstream from the initiation codon of cecR. Accordingly, a strong promoter was detected using an in vitro transcription

Fig. 1. Genomic SELEX screening of CecR-binding sequences on the E. coli genome. Genomic SELEX screening of DNA-binding sequences was performed for CecR (renamed YbiH), an as yet uncharacterized TerR-family TF of E. coli, using purified C-terminal His-tagged CecR and a library of DNA segments from the E. coli K-12 genome. A single strong binding site was identified within a 228 bp spacer between divergent transcription units, the ybiH-ybhFSR operon and the rhlE gene.
Role of CecR in regulation in vitro of the bidirectional transcription from the cecR and rhlE promoters

For identification of the functional role of the constitutive promoter detected in the presence of RNAP RpoD holoenzyme alone (see Fig. 4a, lane RNAP alone), we next performed in vitro transcription using the RNAP RpoD holoenzyme that was reconstituted in vitro from the sigma-free purified RNAP core enzyme and twofold molar excess of the purified RpoD sigma subunit, and three templates, 615 bp template-A, 473 bp template-B and 868 bp template-C (Fig. 2b), all including a 228 bp spacer. Using template-A, a transcript of about 400 nt in size was identified, but in the presence of increasing concentrations of CecR, this transcript decreased (Fig. 2a, DNA-1 = template-A), supporting the repression of cecR promoter by CecR. Besides the clear transcript from the cecR promoter, a low level of small RNA of approximately 40 nt in size was detected, which might represent rhlE transcript (Fig. 2a, DNA-1 = template-A).

Using 473 bp template-B, a transcript of about 250 nt in size was detected (Fig. 2a, template-B), which was predicted to be the transcript from the cecR promoter (Fig. 2b, DNA-2 = template-B). This transcript was also reduced in the simultaneous presence of CecR protein. The small 40 nt RNA detected with the use of template-B was identical to that identified with template-1 (Fig. 2a, DNA-2 = template-B). Finally, transcripts were analysed with the use of 868 bp template-C (Fig. 2b, template-C). The predicted cecR RNA of about 250 nt in size was identical to that detected with use of template-B, in good agreement with assay, which directs transcription toward the cecR operon (see below for in vitro transcription assay). By gel shift assay, it is often difficult to identify weak promoters without a clear consensus promoter sequence for activator-dependent promoters (Shimada et al., 2014). Thus we cannot rule out the possibility that an activator-dependent promoter for the rhlE promoter was not detected in this footprinting assay (see below for the regulation mode of the rhlE promoter).

This 20 bp CecR-box sequence is located immediately downstream of the cecR promoter, completely overlapping with the RNAP RpoD holoenzyme-binding sequence of 79 bp in length. The overlapping organization of RNAP- and CecR-binding signals indicates that the latter functions as a repressor. In good agreement with this prediction, the binding of RNAP to this cecR promoter was completely prevented by the addition of CecR (Fig. 4a, lane CecR plus RNAP). The reporter assay of the cecR promoter also supported the repression mode of the cecR promoter by CecR itself (see below).

Fig. 2. Transcription in vitro of the cecR and rhlE genes. Transcription in vitro was performed using three different templates [template-A, template-B and template-C in (b)] and the reconstituted RNAP RpoD holoenzyme. Template-A directed transcription from the cecR promoter while both template-B and template-C directed transcription from both the cecR and the rhlE promoters. The synthesis of cecR RNA was significantly decreased in the simultaneous presence of CecR protein. The level of rhlE transcription by RNAP alone was low and the influence of CecR addition on the rhlE promoter was not clear under the conditions employed. Templates used were 0.1 pmol per assay, while CecR protein used was: 0, 0.1, 0.5 and 1.0 pmol for lanes 1–4.

Fig. 3. Gel shift assay of CecR–DNA complex formation. Increasing amounts of the purified CecR was mixed, in a total of 0.015 ml of gel-shift buffer, with 1.0 pmol of a DNA probe corresponding to a 311 bp reference probe corresponding to an N-terminal segment of the cecR gene (a) or 228 bp spacer sequence between the cecR operon and the rhlE gene (b). CecR added was: lane 1, 0 pmol; lane 2, 0.03 pmol; lane 3, 0.10 pmol; lane 4, 0.30 pmol; lane 5, 1.0 pmol; lane 6, 3.0 pmol; and lane 7, 10 pmol. A single band of CecR–DNA complex was identified.
the gene organization along the templates used. This cecR RNA also decreased in the presence of CecR addition (Fig. 2a, DNA-3 = template-C).

With template-C, the predicted small rhlE RNA identified with use of template-A and template-B disappeared but, instead, three weak bands of long RNA were detected, of which the longest RNA represents a product of end-to-end transcription of this template (Fig. 2a, DNA-3 = template-C). One of the other two RNA bands might be a transcript from the rhlE promoter. In the absence of strong promoter sequence, transcription initiation in vitro often takes place at multiple sites. By DNase footprinting assay, the region protected by RNAP RpoD alone was a constitutive promoter of 79 bp in length (a), which corresponds to −4 to −82 with respect to the cecR initiation codon as indicated by dotted underline in (b). The CecR-binding sequence completely overlaps with the RpoD holoenzyme-binding sequence. In the presence of excess CecR, the binding of RNAP is blocked (a), implying that CecR represses the constitutive promoter.

**Role of CecR in regulation in vivo of the bidirectional transcription from the cecR and rhlE promoters: reporter assay**

To confirm the regulatory role in vivo of CecR of the bidirectional transcription of the cecR-ybhGFSR operon and the rhlE gene, we employed the reporter assay system using the bacterial luciferase encoded by the luxC gene (Blouin et al., 1996; Yamanaka et al., 2014). The full-sized 288 bp intergenic spacer of the bidirectional transcription units was fused, in both directions, with the luxC gene (Fig. 5). Each of these promoter assay vectors was transformed into wild-type BW25113 and its otherwise identical cecR knock-out mutant JW1788. Activity of the cecR promoter was markedly enhanced for the cecR knock-out mutant as measured by luciferase activity (Fig. 5a), implying the autonomous repression of the cecR promoter by the CecR regulator. This enhancement of the cecR promoter was in particular significant when the transformant was grown in a poor M9-glucose medium, suggesting derepression of the cecR-ybhGFSR operon under starved conditions.
Regulatory role in vivo of CecR: PM analysis

For identification of the regulatory function of CecR, we employed the PM system (Biolog), which allows the growth of a test bacterial strain under a total of 1920 different conditions using a total of 20 plates (PM1 to PM20), each including 96 different culture conditions (Bochner et al., 2001; Ogasawara et al., 2015). Here we performed the PM assay for a pair of wild-type BW25113 and the otherwise identical cecR-deleted mutant JW1788 from the Keio collection. Cell growth was monitored every 15 min for 24 h (96 time points) by measuring the cell-density-dependent increase in respiration. Phenotypic identification using this PM system is, however, very tricky, showing a wide range of data fluctuation. After PM screenings for more then 30 different E. coli mutants from the Keio collection (Baba et al., 2006), each lacking a specific TF gene, we have identified a set of compounds that always exhibited high levels of fluctuation, such as Tweens and potassium superoxide, giving a high level of background noise in the PM assay (A. Ishihama, unpublished data). This knowledge was used to increase the data quality. A significant difference was identified in cell growth with use of PM plates 11–20 for the sensitivity test to various toxic compounds. The compounds that exhibited different patterns of cell growth between the wild-type E. coli and the cecR mutant are summarized in Fig. 6. Growth of the cecR mutant was better than the wild-type parent in the presence of nalidixic acid, polymyxin, diamine and D-serine (Fig. 6, green shading). By contrast, the cecR mutant was more sensitive than the wild-type parent to cefazolin (a first-generation cephalosporin), chloramphenicol, benzethonium, spiramycin, lithium chloride, trimidazole, guanazole and chlorhexidine (Fig. 6, red shading).

To confirm the sensitivity of the cecR mutant to these drugs, we next examined growth of the cecR mutant in liquid culture containing each of these drugs (data not shown). Among the whole set of candidate antibiotics examined, a significant reduction in the drug sensitivity of the cecR mutant was observed for two antibiotics, cefazolin and chloramphenicol. The PM assay indicated involvement of CecR in the regulation of the gene(s) that participate in the sensitivity control of two antibiotics, cefazolin and chloramphenicol. The cecR mutant was also more sensitive to cefoxitin (a second-generation cephalosporin) and cefoperazone (a third-generation cephalosporin). In this study, we used cefoperazone as a representative of the cephalosporin group antibiotics. We then focused on the two antibiotics cefoperazone and chloramphenicol for detailed analysis of the regulatory role of CecR.

Regulatory role of CecR (YbiH) in antibiotic sensitivity

The possible influence of two antibiotics, cefoperazone and chloramphenicol, on the growth of E. coli wild-type BW25113 and the otherwise identical cecR mutant JW1788 was examined in liquid culture. In the presence of various concentrations of these drugs, cell growth was monitored for various times in both poor and rich media. The effect of the two antibiotics was more pronounced in M9-glucose medium, so detailed analysis was performed using this poor medium. Growth of the mutant lacking CecR was markedly reduced in the presence of cefoperazone at 2.5 µg ml⁻¹ and above (Fig. 7ai–iii), and completely inhibited at 10 µg ml⁻¹ and above (Fig. 7iv–v). Likewise, growth of this cecR mutant was inhibited in the presence of chloramphenicol at 5 µg ml⁻¹ and above (Fig. 7vi–v). These observations indicate the involvement of CecR in the sensitivity control.
to these antibiotics supposedly through regulating the expression level of one or more of its regulatory targets.

The cecR operon includes the gene encoding an inner membrane protein YbhG and three genes encoding an ABC-type transporter YbhFSR (see Fig. 1), both of which might be involved in transport of the two antibiotics chloramphenicol and cefoperazone. If this is the case, the mutants lacking the genes encoding these membrane proteins should exhibit altered sensitivity to these drugs. To test this possibility, growth of the mutants lacking ybhG (JW0779) and ybhF (JW5104) was monitored in the presence and absence of these drugs. In the presence of cefoperazone at 2.5 µg ml\(^{-1}\), the growth of cecR, ybhG and ybhF mutants was significantly reduced compared with the wild-type (Fig. 7b, c) even though the growth rate was essentially the same for all these test strains in the absence of antibiotics. The decrease in growth rate in the presence of cefoperazone was highest for the ybhF gene encoding the ATP-binding component of the YbhFSR ABC-type transporter, suggesting the role of YbhFSR in efflux of cefoperazone. By contrast, in the presence of chloramphenicol at 5.0 µg ml\(^{-1}\), growth of the ybhG mutant was significantly reduced compared with the wild-type and the cecR and ybhF mutants (Fig. 7c), implying involvement of the YbhG inner membrane protein in the sensitivity control to chloramphenicol, possibly at the efflux pathway. On the basis of these observations, we predicted involvement of the YbhFSR transporter in the sensitivity to cefoperazone, and of the inner membrane protein YbhG in the sensitivity to chloramphenicol.

**DISCUSSION**

**Genomic SELEX screening of regulatory targets of CecR**

For identification of regulatory targets of all seven RNAP sigma subunits and approximately 300 TFs in *E. coli*, we developed the genomic SELEX screening system (Shimada et al., 2005), which is, in particular, useful for the identification of regulatory targets of as yet uncharacterized TFs. For the identification of recognition sequences of test regulatory proteins with high accuracy, we employed both SELEX-clos (cloning–sequencing) and SELEX-chip (tiling array analysis) procedures. In the case of CecR (renamed YbiH), more than 90% independently isolated SELEX clones carried the spacer sequence between the cecR and rhlE genes as detected by SELEX-clos whereas a single high-level peak was identified in the same region of the *E. coli* genome by SELEX-chip (see Fig. 1).
Using the genomic SELEX screening system, we have carried out a systematic screening of regulatory targets for more than 200 *E. coli* TFs (Ishihama et al., 2016). The number of regulatory targets was found to range from a single target up to approximately 1000 (Ishihama et al., 2016). One surprising finding is that even though most of the *E. coli* TFs have long been believed to regulate only one specific target gene or operon (Ishihama, 2010, 2012), the single-target TF is, however, rather rare, including CecR (this study), NimR (renamed YeaM) (Ogasawara et al., 2015), BetI, NanR and UlaR (Ishihama et al., 2016).

**Role of ABC-transporter YbHFSR in cefoperazone sensitivity**

Five families of the bacterial drug extrusion transporter have been identified based on sequence similarity (Paulsen et al., 1996; Putman et al., 2000; Levy, 2002), consisting of the MFS (major facilitator superfamily), SMR (small multidrug resistance), RND (resistance nodulation cell division), ABC (ATP-binding cassette) and MATE (multidrug and toxic compound extrusion) families. In *E. coli*, a total of 37 putative drug transporter genes (19 MFS, three SMR, seven RND, seven ABC and one MATE) were found from the sequence annotation. ABC transporters constitute one of the largest families of membrane proteins (Theodoulou & Ken, 2015). Bacterial ABC transporters play a major role in virulence and drug resistance.

YbhRSF is one of six uncharacterized ABC transporters in *E. coli* (Moussatova et al., 2008). Until now, nothing was known on the function of YbhFSR (YbhF, ATP-dependent component; YbhRS, membrane components) (Daley et al., 2005). In this study, we identified that the sensitivity to cefoperazone increased for mutants lacking cecR (the regulator of the ybhFSR operon) and ybhF (Fig. 7), implying

![Fig. 7. Influence of cefoperazone and chloramphenicol on cell growth. (a) *E. coli* wild-type BW25113 and its cecR mutant JW1788 were grown in M9-glucose medium containing various concentrations of cefoperazone (*v*: 0, 1.0, 2.5, 10 and 100 µg ml⁻¹). The cecR mutant did not grow in the presence of cefoperazone above 10 µg ml⁻¹. (b) *E. coli* wild-type BW25113 and its cecR mutant JW1788 were grown in M9-glucose containing various concentrations of chloramphenicol (*v*: 0, 0.50, 1.0, 2.5 and 5.0 µg ml⁻¹). The cecR mutant did not grow in the presence of chloramphenicol above 5.0 µg ml⁻¹. (c) *E. coli* wild-type BW25113 and a group of the otherwise isogenic mutants, each lacking cecR or one of its regulatory targets, ybhG and ybhF, were grown in M9-glucose medium in the absence (i) or presence of cefoperazone (ii) or chloramphenicol (iii). Cell growth was monitored for 25 h by measuring the turbidity at 600 nm.](http://mic.microbiologyresearch.org)
that the ABC-type YbhRSF transporter is involved in efflux of cefoperazone (a third-generation cefalosporin). The β-lactam group antibiotics inhibit the synthesis of the peptidoglycan (PG) layer that constitutes the bacterial cell wall. The final step of PG synthesis is facilitated by a transpeptidase known as penicillin-binding protein (PBP), the target of β-lactam action. PG is also under dynamic recycling depending on growth conditions, 0in which PgrR (renamed YcjZ) plays a switching role by controlling a set of genes for PG degradation and recycling (Shimada et al., 2013).

One major regulatory role of TetR-family TFs is the control of a large set of genes involved in drug efflux (Ramos et al., 2005). The major multidrug efflux systems in E. coli include RND-type AcrAB–TolC complex (Ma et al., 1994, 1996) and RND-type AcrEF–TolC complex (Hirakawa et al., 2008). Transcription of acrAB is under the control of TetR-family AcrR (acriflavin resistance regulator) (Ma et al., 1994, 1996; Lee et al., 2014) while transcription of acrEF is regulated by TetR-family EnvR (Hirakawa et al., 2008). Likewise, transcription of ybhF gene was found to be under the control of TetR-family CecR.

Role of YbhG membrane protein in chloramphenicol sensitivity

Chloramphenicol is an effective antibiotic against a wide variety of Gram-positive and Gram-negative bacteria. Once chloramphenicol, a toxic antibiotic, is taken up into E. coli cells, it is converted to a less toxic acetylated form by the action of chloramphenicol acetyltransferase or the cat gene product. This is the major resistance mechanism to chloramphenicol, and the acetylated chloramphenicol is further decomposed or secreted into the periplasm and ultimately out of the cells through an efflux pump. The entry and efflux of chloramphenicol are both targets affecting the control of TetR-family AcrR (acriflavin resistance regulator) (Ma et al., 1994, 1996; Lee et al., 2014) while transcription of acrEF is regulated by TetR-family EnvR (Hirakawa et al., 2008). Likewise, transcription of ybhF gene was found to be under the control of TetR-family CecR.

Role of RNA helicase RhlE in chloramphenicol resistance

The participation of CecR on regulation of the rhlE gene was suggested by the in vivo reporter assay (see Fig. 5). Chloramphenicol binds to the 50S ribosomal subunit and inhibits protein chain elongation by inhibiting peptidyl transferase activity (Wilson, 2009). Chloramphenicol binds to 50S ribosome at a molar ratio of 1:1 (Wolf & Hahn, 1965). Biochemical and crystallographic studies have identified the binding of chloramphenicol to the A site of peptidyl transferase (Schlunzen et al., 2001; Hansen et al., 2003; Bulkley et al., 2010; Dunkle et al., 2010). High-resolution X-ray structures of the 50S ribosome–chloramphenicol complex indicated its binding site on segments of 23S rRNA at the peptidyl transferase cavity (Schlunzen et al., 2001). The bases of 23S rRNA A2062, C2452 and U2506 have been suggested to be involved in the interaction with chloramphenicol (Mankin & Garrett, 1991; Dunkle et al., 2010).

During the assembly of ribosomes, RNA helicases have been suggested to be involved in folding of rRNA for effective interaction with ribosomal proteins. E. coli contains five or six members of the the DEAD-box helicase family (SrmB, CsdA, DbpA, RhlE, RhlB and RhlF) (Iost & Dreyfus, 2006), which have been implicated directly in ribosome biogenesis (Charollais et al., 2003; Jain, 2008; Jagessar & Jain, 2010). For instance, in the early step of 50S assembly, SrmB was identified to play a role in the early steps of ribosome biogenesis, by forming a quaternary complex with the ribosomal proteins L4 and L24 (Trubetskoy et al., 2009). The cold-shock RNA helicase, CsdA, also participates in the biosynthesis of 50S ribosome (Charollais et al., 2004). In the presence of chloramphenicol, expression of the rhlE gene is activated so as to produce more RNA helicase, probably as a defence against the inhibitory action of chloramphenicol on rRNA. The finding of an involvement of chloramphenicol in activation of the rhlE gene might add to our understanding of the connection between the inhibitory function of chloramphenicol in ribosome function and the involvement of RNA helicase in ribosome biogenesis.

ACKNOWLEDGEMENTS

We thank Ayako Kori for preparation of CecR protein and SELEX screening, Kiyo Hirao for SELEX-clos analysis, and Kaoru Yamazaki, Shuto Suzuki and Syuto Saito for PM screening. This work was supported by Grant-in-Aid 21241047 from the Ministry of Education, Culture, Sports, Science and Technology of Japan to A.I.; MEXT Cooperative Research Program of Network Joint Research Center for Materials and Devices to A.I., K.Y. and T.S.; and Nano-Biology Project Fund from Micro-Nano Technology Research Center of Hosei University to A.I. and K.Y.

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Edited by: D. Grainger