Cooperative regulation of the common target genes between H$_2$O$_2$-sensing YedVW and Cu$^{2+}$-sensing CusSR in *Escherichia coli*

Hiroyuki Urano,$^1$ Yoshinoma Umezawa,$^2$ Kaneyoshi Yamamoto,$^{2,3}$ Akira Ishihama$^{2,3}$ and Hiroshi Ogasawara$^1$

$^1$Research Center for Human and Environmental Sciences, Shinshu University, Ueda, Nagano 386-8567, Japan
$^2$Department of Frontier Biosciences, Hosei University, Koganei, Tokyo 184-8584, Japan
$^3$Research Center for Micro-Nano Technology, Hosei University, Koganei, Tokyo 184-8584, Japan

YedVW is one of the uncharacterized two-component systems (TCSs) of *Escherichia coli*. In order to identify the regulation targets of YedVW, we performed genomic SELEX (systematic evolution of ligands by exponential enrichment) screening using phosphorylated YedW and an *E. coli* DNA library, and identified YedW-binding sites within three intergenic spacers, *yedW*-hiuH, *cyoA*-ampG and *cusR*-cusC, along the *E. coli* genome. Using a reporter assay system, we found that transcription of *hiuH*, encoding 5-hydroxysourate hydrolase, was induced at high concentrations of either Cu$^{2+}$ or H$_2$O$_2$. Cu$^{2+}$-dependent expression of *hiuH* was observed in the *yedWV*-null mutant, but was reduced markedly in the *cusRS*-null mutant. However, Cu$^{2+}$-induced *hiuH* expression was observed in the *cusRS*-null mutant, but not in the *yedWV*-null mutant. Gel mobility shift and DNase I footprinting analyses showed binding of both YedW and CusR to essentially the same sequence within the *hiuH* promoter region. Taken together, we concluded that YedVW and CusSR formed a unique cooperative TCS pair by recognizing and regulating the same targets, but under different environmental conditions – YedVW played a role in H$_2$O$_2$ response regulation, whilst CusSR played a role in Cu$^{2+}$ response regulation.

**INTRODUCTION**

In order to adapt to stresses in nature, bacteria carry sophisticated regulation systems to control the expression pattern of the genome as a whole. Genome expression in the model prokaryote *Escherichia coli* is determined mainly at the stage of transcription by controlling the distribution of a limited number of RNA polymerases through interaction with two groups of regulatory proteins, i.e. seven species of the sigma subunit with promoter recognition activity and ~300 species of transcription factors (TFs) (Ishihama, 2010, 2012). Most of these TFs carry, within single molecules, both a functional domain for sensing effector signals and environmental conditions, and a DNA-binding regulatory domain for controlling transcription. In the case of two-component systems (TCSs), however, the sensor domains and the transcription regulation domains are divided into two proteins, i.e. a membrane-associated sensor kinase and a cytoplasmic response regulator. *E. coli* contains a total of ~30 TCS pairs ( Mizuno, 1997; Oshima et al., 2002; Yamamoto et al., 2005).

The intracellular concentration of copper in *E. coli* is tightly maintained at a fixed level irrespective of variations in the copper level in the environment. To maintain copper homeostasis within *E. coli* cells, three kinds of TF [two TCS response regulators (CusSR, CpxAR) and one MerR-type TF (CueR)] are involved in the control of the uptake, intracellular delivery and export of copper (Munson et al., 2000; Rensing et al., 2000; Rensing & Grass, 2003; Yamamoto & Ishihama, 2005). In addition, we identified that transcription of the genes encoding an as-yet uncharacterized TCS, YedVW is activated in the presence of copper addition in a CusR-dependent manner (Yamamoto & Ishihama, 2005), implying that the genes under the direct control of YedVW are regulated indirectly by external copper. The sequence of the response regulator YedW is 51 % similar to that of CusR and the sensor YedV phosphorylates not only YedW, but also CusR (Yamamoto & Ishihama, 2005), suggesting cross-talk between YedVW and CusSR TCSs. A common evolutionary origin has been proposed for YedVW and CusSR, with both being maintained over a wide range of bacterial species.

**Abbreviations:** 5-HIU, 5-hydroxyisourate; OHCU, 2-oxo-hydroxy-4-carboxy-5-ureidoimadazline; SELEX, systematic evolution of ligands by exponential enrichment; TCS, two-component system; TF, transcription factor.
Salmonella was used DH5α, BW25113, BW28077, which were constructed using a fusion that functions as the hydrolase of 5-hydroxyisourea (5-HIU), a degradation product of uric acid, in the purine catabolism pathway (Lee et al., 2005; Hennebry et al., 2012). The possible influence in vivo of YedW on the predicted promoters for the target gene promoters was examined using the LacZ reporter assay. After searching for an effector(s) affecting the YedV sensor, we identified H$_2$O$_2$ as an inducer candidate, which is generated in the process of 5-HIU production by H$_2$O$_2$– one of the regulation targets of YedW. Furthermore, we identified the H$_2$O$_2$-dependent regulation of YedW target genes. YedW and CusR were found to bind to the same recognition sequences and regulate the same set of genes. Based on these findings, we proposed a novel type of regulation system in which the cooperative regulation of the same targets takes place between H$_2$O$_2$-sensing YedW and copper-sensing CusR, both recognizing essentially the same sequence for DNA binding.

**METHODS**

**Bacterial strains and growth conditions.** E. coli DH5α was used for plasmid amplification. E. coli BL21 was used for expression and purification of YedW and CusR. E. coli BW25113, BW28077 (pCusRS) and BW27550 (pAyedWV) were grown in LB medium at 37°C under aerobic conditions with constant shaking at 160 r.p.m. Cell growth was monitored by measuring OD$_{600}$.

**Plasmid construction.** For the construction of the hiiH–lacZ reporter plasmid, a DNA fragment containing the hiiH promoter region was prepared by PCR using BW25113 genome DNA as a template and a pair of gene-specific primers (hiiH-EcoRI-F and hiiH-BamHI-R, Table 1). 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.

**Purification of YedW and CusR proteins.** For purification of YedW and CusR, we used overexpression plasmids pYedW (renamed pKH120-1) (Yamamoto et al., 2005a) and pCusR. E. coli BL21 transformants were grown in LB broth in the presence of 100 μg ampicillin ml$^{-1}$, and expression of His-tagged YedW and CusR was induced in the mid-exponential phase by adding 1 mM IPTG. After 3 h induction, cells were harvested and protein purification was carried out according to our standard laboratory procedures (Yamamoto et al., 2005). In brief, lysozyme-treated cells were sonicated in the presence of 100 mM PMSF. After centrifugation of cell lysate at 15 000 r.p.m. for 20 min at 4°C, the resulting supernatant was mixed with 2 ml 50% Ni-NTA agarose resin (Qiagen) and loaded onto a column. After washing with 10 ml lysis buffer (50 mM Tris/HCl, pH 8.0 at 4°C, 100 mM NaCl), the column was washed with 10 ml washing buffer (50 mM Tris/HCl, pH 8.0 at 4°C, 100 mM NaCl). Proteins were then eluted with 2 ml elution buffer (200 mM imidazole, 50 mM Tris/HCl, pH 8.0 at 4°C, 100 mM NaCl) and dialysed against storage buffer (10 mM Tris/HCl, pH 7.6, 200 mM KCl, 10 mM MgCl$_2$, 0.1 mM EDTA, 1 mM DTT, 50%, v/v, glycerol).

**Genomic SELEX screening of YedW-binding sequences.** Genomic SELEX screening of YedW-binding sequences was carried out using an improved procedure as described previously (Shimada et al., 2005; Ogasawara et al., 2012). A mixture of DNA fragments of the E. coli K-12 W3110 genome was prepared after sonication of purified genome DNA and cloned into a multi-copy plasmid, pBR322. In each SELEX screening, the DNA mixture was regenerated by PCR (12–18 cycles). For SELEX screening, 5 pmol of the mixture of DNA fragments and 10 pmol His-tagged YedW were mixed in a binding buffer (10 mM Tris/HCl, pH 7.8 at 4°C, 3 mM magnesium acetate, 150 mM NaCl, 1.25 mM BSA) and incubated for 30 min at 37°C. The DNA/Tf mixture was applied to a Ni-NTA agarose column and after washing out unbound DNA with the binding buffer containing 10 mM imidazole, the sequences of DNA fragments recovered from the complexes were determined by the SELEX-chip method. For SELEX-chip, PCR-amplified DNA products of the isolated DNA–protein complexes and original DNA library were labelled by Cy3-dCTP and Cy5-dCTP, respectively, using a Klenow random priming system, and then combined. The fluorescently labelled DNA mixtures were hybridized to a DNA microarray consisting of 43 450 different 60-base DNA probes, which were designed to cover the entire E. coli genome at 105 bp intervals (Oxford Gene Technology). The fluorescence intensity of the test sample at each probe was normalized to that of the corresponding peak of the original library. After normalization of each pattern, the Cy5/Cy3 ratio was measured and plotted along the E. coli genome.

**Measurement of promoter activity.** Single-copy gene reporter strains containing hiiH–lacZ were constructed using a JRS45 phage vector, as described previously (Miller, 1972). The recombinant phage containing promoter–lacZ fusion was isolated from the resulting phage lysate, and used to infect E. coli BW25113, BW28077 (pCusRS) and BW27550 (pAyedWV) for screening of kanamycin-resistant and Lac$^+$ colonies. Single-copy promoter–lacZ fusion strains were grown in LB broth, and β-galactosidase activity was measured using ONPG as a substrate, as described previously (Miller, 1972).

**Table 1. Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hiiH-S1RI-F</td>
<td>TTACCCATTTCCCTGGTGCTTTGATTATCT</td>
</tr>
<tr>
<td>hiiH-S1RI-R</td>
<td>GTTTTCTGGTGTTTCGTTCAAATGTGCA</td>
</tr>
<tr>
<td>hiiH-EcoRI-F</td>
<td>AAGTACGAGATATCAGAAAAATAATA</td>
</tr>
<tr>
<td>hiiH-BamHI-R</td>
<td>TGGCGTTTGGATCCGAGATTAAAA</td>
</tr>
<tr>
<td>lacZ-promoter-F</td>
<td>TCACTCTATTAGGACCCCGAGGCTTAA</td>
</tr>
<tr>
<td>lacZ-30R-FITC</td>
<td>FITC-AGGGTTTCCCAGTCACGAGTTGTAACAC</td>
</tr>
</tbody>
</table>
DNA polymerase (TaKaRa). The promoter region was generated by PCR using a pair of primers (hiuH-EcoRI-F and lac30R-FITC, Table 1) and pRSShiuH as a template, and Ex Taq DNA polymerase (TaKaRa). The FITC-labelled probe of the lac2 promoter region was generated by PCR amplification using a pair of primers (lacZ-promoter-F and lac30R-FITC, Table 1) and BW25113 genome DNA as a template, and Ex Taq DNA polymerase (TaKaRa). The 32P-labelled probe was generated by PCR amplification of the hiuH promoter region using a pair of primers (hiuH-S1RI-F and 32P-labelled hiuH-S1RI-R, Table 1) and BW25113 genome DNA as a template, and Ex Taq DNA polymerase (TaKaRa). PCR products with FITC or 32P at their termini were purified by PAGE. For gel mobility shift assays, mixtures of the FITC- or 32P-labelled probes and purified TFs were incubated at 37°C for 30 min in 12 μl gel shift buffer (10 mM Tris/ HCl, pH 7.8 at 4°C, 150 mM NaCl, 3 mM magnesium acetate). After addition of a DNA dye solution, the mixture was analysed by 6% PAGE. Fluorescently labelled DNA in gels was detected using a Typhoon Trio variable-mode imager (GE Healthcare).

DNase I footprinting analysis. Labelling of probe DNA with FITC or 32P was performed as described previously (Yamamoto & Ishihama, 2005; Ogasawara et al., 2010). Each 1.0 pmol FITC-labelled DNA probe was incubated at 37°C for 30 min with various amounts of CusR or YedW in 25 μl DNase I footprinting solution (10 mM Tris/HCl, pH 7.8, 150 mM NaCl, 3 mM magnesium acetate, 5 mM CaCl2, 25 μg BSA ml⁻¹). After incubation for 30 min, DNA digestion was initiated by the addition of DNase I (TaKaRa). The reaction was terminated by the addition of 25 μl phenol. Phenol-treated samples were precipitated with ethanol, dissolved in formamide dye solution and analysed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea using a slab gel electrophoresis system (DSQ-2000LI; Shimadzu).

Purification of total RNA. Total RNA was extracted from mid-exponential-phase cultures (OD600 0.5–0.6) of E. coli BW25113 and its isogenic ΔcusRS mutant strain grown in LB medium with or without the addition of 0.5 mM CuSO4, as described previously (Yamamoto & Ishihama, 2005).

S1 nuclease assays. S1 nuclease assays were carried out as described previously (Yamamoto & Ishihama, 2005). The 32P-labelled probe was generated by PCR amplification of the hiuH promoter region using a pair of primers (hiuH-S1RI-F and 32P-labelled hiuH-S1RI-R, Table 1) and BW25113 genome DNA as a template, and Ex Taq DNA polymerase (TaKaRa). Mixtures of the 32P-labelled probe (10⁸ c.p.m.) and total RNA (100 g) were incubated for 10 min at 75°C for denaturation, and then incubated at 37°C overnight for hybridization. After digestion with S1 nuclease (TaKaRa) at 37°C for 30 min, undigested products were extracted with phenol, precipitated with ethanol and analysed by electrophoresis on polyacrylamide gels containing 8M urea. The intensity of undigested probe bands on gels was measured using a Typhoon Trio variable-mode imager (GE Healthcare).

RESULTS

Screening of the regulation targets of YedW by genomic SELEX

To identify a set of regulation target genes under the direct control of the uncharacterized YedW, we searched for the whole set of YedW recognition sequences on the E. coli genome using an improved genomic SELEX screening system (Shimada et al., 2005; Ogasawara et al., 2012). Up to the present time, the genomic SELEX screening system has been employed successfully for the identification of the whole set of promoters by each of seven sigma subunits and the whole set of regulation targets by each of >200 DNA-dependent TFs (Ishihama, 2012). In this study, purified His-tagged YedW was mixed with a collection of E. coli genome fragments of 200–300 bp in length in the presence of acetyl phosphate, which is expected to phosphorylate YedW in the absence of YedV kinase. Phosphorylated YedW-bound DNA fragments were affinity-purified for the identification of YedW recognition sequences. The original mixture of genomic DNA fragments used in this genomic SELEX screening formed smeared bands on PAGE, but after four cycles of genomic SELEX, phosphorylated YedW-bound DNA fragments formed several sharp bands on PAGE (data not shown), indicating further enrichment of some DNA fragments with high affinity to YedW. To identify the whole set of targets under the direct control of YedW, we then subjected the mixture of genomic SELEX fragments to DNA-chip analysis using an E. coli tiling array. The phosphorylated YedW-bound DNA fragments were labelled with Cy5, whilst the original DNA library was labelled with Cy3. The mixtures were hybridized with the DNA tiling microarray (Oxford Gene Technology) and the fluorescence intensities bound on each probe were measured. For identification of YedW-binding sites, the Cy5/Cy3 ratio was plotted along the corresponding position on the E. coli genome (Fig. 1a).

By setting the cut-off level at 4 and selecting peaks showing high-level binding of YedW for at least two adjacent probes, a total of three YedW-binding sites were identified within intergenic spacer regions cyoA<<-ampG, yedW<<-hiuH and cysH<<-cysC (Fig. 1b) (note that TF-bound DNA fragments of 200–300 bp in length should bind to at least two adjacent probes and thus peaks of one-probe binding were judged as non-specific noise). Based on the organization of flanking genes, five operons were predicted to be under the direct control of YedW, i.e. cyoABCDE, yedWV, hiuH, cusRS and cusCFBA. Taken together, we predicted that the promoters located near the YedW-binding sites identified in SELEX-chip were the regulation targets of phosphorylated YedW. This newly identified YedW regulon includes two known targets, cusRS and cusCFBA, of the Cu²⁺-sensing CusR regulon (Yamamoto & Ishihama, 2005). The hiuH gene encoding the hydrolase of 5-HIU, a product of the purine degradation pathway, is located next to the divergently transcribed yedW gene. As the set of TFs and their regulation target genes are often organized side-by-side on the E. coli genome, detailed analysis of the regulatory role of YedW was performed focusing on the divergently transcribed hiuH gene.

Identification of YedW-binding sites on the predicted target promoters

To examine the predicted regulation targets of phosphorylated YedW, we performed gel mobility shift assays. First, 32P-labelled hiuH promoter fragments were mixed with...
increasing concentrations of YedW in the presence or absence of acetyl phosphate, and immediately subjected to PAGE after incubation. The gel shift pattern of the $^{32}$P-labelled $hiuH$ promoter fragment indicated the decrease of free DNA concomitant with the increase of YedW addition (Fig. 2a, left panels). At high concentrations of YedW, the initial $hiuH$ probe–YedW complex was further shifted, being trapped at the top of the gel. Even though some response regulator proteins, such as CheY, PhoB and OmpR, are known to be phosphorylated in vitro with acetyl phosphate (Lukat et al., 1992; McCleary & Stock, 1994), phosphorylation in vitro of YedW by acetyl phosphate has not yet been proven. However, the supershift of the $hiuH$ promoter probe was more significant in the presence of acetyl phosphate (Fig. 2a, left panels), indicating cooperative interaction of the activated YedW at high protein concentrations.

To identify the YedW-binding sequences, we next performed DNase I footprinting analysis of YedW-bound $hiuH$ DNA fragments (Fig. 2b, left panel). The region protected by YedW from DNase I digestion included a 28 bp sequence between −40 and −67 from its transcription start position. This sequence contained an 18 bp palindromic sequence tentatively referred to the YedW-box (Fig. 2b, right panel). Using this 28 bp sequence and the BioProspector program (http://ai.stanford.edu/~xsliu/BioProspector/), we searched for a consensus sequence recognized by YedW within all the spacer sequences with YedW-binding activity and identified the 18 bp sequence motif of the YedW-box (Fig. 3). After genome-wide searching of the YedW-box sequence by setting two mismatches, we found a number of CATNAC-AANNTTGTAATG-like sequences on the $E. coli$ genome (data not shown). However, we failed to detect significant peaks of YedW binding around these sequences. To our surprise, this YedW-box sequence completely agreed with the CusR-binding sequence on the $cusC$ promoter region (Yamamoto & Ishihama, 2005). This finding implied that the $hiuH$ promoter may be regulated by the copper-sensing CusSR TCS.

**Identification of the CusR-binding site on the $hiuH$ promoter**

To test the possibility of CusR binding to the $hiuH$ promoter, we performed gel mobility shift assays on the binding of phosphorylated CusR to the $hiuH$ promoter probe. In the presence of increasing concentrations of phosphorylated CusR, the $^{32}$P-labelled $hiuH$ promoter probe was converted to form the CusR complex (Fig. 2a, right panel). To identify the sequence recognized by CusR, we next performed DNase I footprinting analysis. A
Fig. 2. Analysis of YedW- and CusR-binding sites on the hiuH promoter. (a) Gel mobility shift assays. (Left panels) Gel mobility shift assay of YedW binding to the hiuH promoter probe. \(^{32}\)P-labelled hiuH promoter probe was mixed with increasing amounts of YedW in the absence (YedW lanes) or presence (YedW-P lanes) of acetyl phosphate. After incubation at 37 °C for 30 min, the mixtures were subjected to PAGE. The amounts of YedW added for lanes 1–5 were 0, 1.25, 2.5, 5 and 10 pmol, respectively. (Right panel) Gel mobility shift assay of CusR binding to the hiuH promoter probe. FITC-labelled hiuH promoter probe was mixed with increasing amounts of purified CusR and subjected to PAGE after incubation at 37 °C for 30 min. The amounts of CusR added for lanes 1–6 were 0, 12.5, 25, 50, 100 and 200 pmol, respectively. A fluorescent DNA probe containing the lacZ promoter was added to each reaction as a reference control. (b) DNase I footprinting analysis of the YedW-binding site on the hiuH promoter region. (Left panel) The \(^{32}\)P-labelled hiuH promoter segment was incubated in the absence or presence of increasing concentrations of purified YedW and then subjected to DNase I footprinting analysis. The amounts of YedW added for lanes 1 and 2 were 0 and 40 pmol, respectively. Lane M indicates the Maxam–Gilbert sequencing reaction ladder. The black bar on the right indicates the YedW-binding region. (Right panel) The location of the YedW-binding region is shown on the sequence between the translation initiation sites of yedW and hiuH. (c) DNase I footprinting analysis of the CusR-binding site on the hiuH promoter region. (Left panel) The FITC-labelled hiuH promoter segment (1 pmol) was incubated in the absence or presence of increasing concentrations of purified CusR and then subjected to DNase I footprinting analysis. The amounts of CusR added for lanes 1–5 were 0, 12.5, 25, 50, 100 and 200 pmol, respectively. Lanes A, T, G and C represent the respective sequence ladders. The black bar on the right indicates the CusR-binding region. (Right panel) The location of the CusR-binding region is shown on the sequence between the translation initiation sites of yedW and hiuH.
sequence of 33 bp was protected by phosphorylated CusR from DNase I digestion (Fig. 2c, left panel).

The CusR-binding sequence on the hiuH promoter agreed (Fig. 2c, right panel) with that identified previously on the cusC promoter (Yamamoto & Ishihama, 2005). The CusR-binding sequence (CusR-box) completely overlapped with the YedW-box sequence (Fig. 3). This finding indicated that two TCS regulators, YedW and CusR, bound to the same sequence and regulated the same target hiuH promoter. The physiological implications of this unique gene organization of two TFs will be discussed below.

**Regulation in vivo of the hiuH promoter by YedVW**

As HiuH is considered to be involved in the degradation pathway of uric acid to allantoin, the possible influence of uric acid was tested, although no significant difference was found in the growth rates between the WT and yedWV mutant in the presence or absence of uric acid (data not shown). During the oxidation of uric acid, 

\[ \text{H}_2\text{O}_2 \]

is generated with the production of 5-HIU. We thus examined the possible influence of 

\[ \text{H}_2\text{O}_2 \]

on hiuH expression. In order to determine YedW-dependent regulation in vivo of the hiuH promoter, we employed the S1 nuclease assay. For this purpose, total RNA was isolated from E. coli cells and subjected to S1 nuclease assay using a hiuH-specific probe consisting of the sequence between −181 and +121 from its translational start position. When 

\[ \text{H}_2\text{O}_2 \]

was added for 30 min in the mid-exponential phase of WT E. coli, the level of hiuH mRNA increased concomitant with the increase in 

\[ \text{H}_2\text{O}_2 \]

concentration, at least up to 6 mM (Fig. 4a). To identify the maximum level of induction, hiuH mRNA was determined at various times after 

\[ \text{H}_2\text{O}_2 \]

addition (Fig. 4b). The induction level was maximum at 30 min, but thereafter decreased gradually.

Under the conditions (6 mM 

\[ \text{H}_2\text{O}_2 \]

addition for 30 min) giving the maximum induction of hiuH transcriptions, the growth rate did not change significantly between the WT and mutants defective in either CusSR and YedVW (data not shown). Activation of hiuH expression was not observed in the yedWV mutant (Fig. 4c, lanes 5 and 6), but the 

\[ \text{H}_2\text{O}_2 \]

-dependent expression of hiuH mRNA in the yedWV mutant was as high as that in the WT (Fig. 4c, lanes 3 and 4). These findings indicated that YedVW, but not CusSR, was involved in 

\[ \text{H}_2\text{O}_2 \]

-dependent induction of hiuH transcription.

**Regulation in vivo of the hiuH promoter by CusSR**

To confirm the CusSR-dependent regulation in vivo of the hiuH promoter, we also determined the level of hiuH mRNA in the presence or absence of 

\[ \text{Cu}^{2+} \]

in WT E. coli cells were grown in LB medium, and after addition of 0.25 mM 

\[ \text{CuSO}_4 \]

total RNA was isolated and subjected to S1 nuclease assay using the same hiuH-specific probe as used for the analysis of the influence of 

\[ \text{H}_2\text{O}_2 \]

. In the presence of 

\[ \text{Cu}^{2+} \]

, the level of hiuH mRNA increased, indicating that 

\[ \text{Cu}^{2+} \]

activated transcription of hiuH (Fig. 5a, lane 2). No detectable level of hiuH mRNA was observed in the absence of 

\[ \text{Cu}^{2+} \]

addition (Fig. 5a, lane 1) and with the 

\[ \text{cusRS} \]

mutant (Fig. 5a, lanes 3 and 4), indicating the involvement of CusR as an activator of hiuH.

Next, we performed an hiuH−lacZ reporter assay in the presence and absence of 

\[ \text{Cu}^{2+} \]

. The expression of hiuH−lacZ in WT E. coli was enhanced by the addition of 0.25 mM 

\[ \text{CuSO}_4 \]

(Fig. 5b, lanes 1 and 2). Essentially the same level of hiuH−lacZ expression was observed in the mutant lacking yedWV (Fig. 5b, lanes 5 and 6), indicating that YedVW was not involved in the 

\[ \text{Cu}^{2+} \]

-dependent activation of the hiuH promoter. Even in the presence of 

\[ \text{Cu}^{2+} \]

, however, hiuH−lacZ expression was not activated in the mutant lacking CusSR (Fig. 5b, lanes 3 and 4). Results of the S1 mapping and reporter assays confirmed the regulation of hiuH by the 

\[ \text{Cu}^{2+} \]

-sensing CusSR.

These observations together indicate that two TCSs, i.e. YedVW and CusSR, form a unique regulation system: (1) the two TCSs recognize the same DNA sequence for binding and cooperate in regulating the same set of target genes and promoters, but (2) the two TCSs recognize different environmental signals for activation – YedVW sensing 

\[ \text{H}_2\text{O}_2 \]

and CusSR sensing 

\[ \text{Cu}^{2+} \].
DISCUSSION

Regulation targets of YedVW

Using genomic SELEX screening, we identified the binding sites of YedW within three intergenic spacer regions on the E. coli genome (Fig. 1) and based on the mapping data at least five operons, cyaABCDE, yedWV, hiuH, cusRS and cusCFBA, these have been predicted to be under the direct control of YedW (Fig. 6). On the E. coli genome, the regulation target genes are generally located next to the regulator genes, but are transcribed in the opposite direction. The regulatory role of YedVW was examined in detail using the hiuH gene as a model system.

In E. coli, unused purines are degraded to uric acid (Vogels & Van der Drift, 1976; Xi et al., 2000), which is then degraded to allantoin via 5-HIU and 2-oxo-hydroxy-4-carboxy-5-ureidoimadeazline (OHCU) (Lee et al., 2005; Hennebry et al., 2012). Under anaerobic conditions, allantoin is further degraded to provide nitrogen sources, whilst under aerobic conditions it is degraded to 3-phosphoglycerate via glyoxylate for energy production. The enzymes and metabolic pathways of purine to uric acid degradation,
and the enzymes for the degradation downstream of allantoin, are well characterized. The TF PurR plays a key role in the regulation of the upstream pathways, whilst AllR plays a major role in the regulation of the downstream pathway (Rintoul et al., 2002; Walker et al., 2006; Hasegawa et al., 2008). The non-enzymic pathway is known as the intermediate pathway for the conversion of uric acid to allantoin, but the enzymes involved in these reaction steps remain unidentified (Xi et al., 2000). Recently, however, the enzyme for the conversion of 5-HIU to OHCU was identified as the hiuH (yedX) gene product (Lee et al., 2005; Hennebry et al., 2012). Urate is generally considered as an antioxidant, but under some circumstances it enhances oxidative stress because urate switches between being a pro-oxidant or antioxidant depending on the Cu²⁺ concentration (Filipe et al., 2002).

In E. coli, HiuH, a transthyretin-like protein, stays in the periplasmic space, as is also the case in Salmonella (Hennebry et al., 2006). Association of the enzymic machinery of the purine metabolism pathway has not been established in the periplasm (Hennebry et al., 2012). One possible role of HiuH is to rapidly reduce possible oxidative damage which might occur if 5-HIU was allowed to spontaneously decompose under normal conditions (Hennebry et al., 2012). When Cu²⁺ ions were present at an equimolar concentration to 5-HIU, the activity of HiuH was strongly inhibited in Salmonella (Hennebry et al., 2012). These results suggested that Cu²⁺-dependent regulation of uric acid metabolism is important for resistance to oxidative stress in E. coli.

Copper is required for the function of several important enzymes in E. coli, including the cytochrome bo ubiquinol oxidase, copper/zinc superoxide dismutase and amine oxidase (Anraku & Gennis, 1987; Cooper et al., 1992; Gort et al., 1999). The newly identified YedW target, i.e. the cyoABCDE operon, encodes cytochrome b₀ ubiquinol oxidase. The YedW-box sequence exists upstream of the cyoA promoter (Fig. 3). The expression level of cyoABCDE significantly decreases after overexpression of YedW (H. Aiba, personal communication), indicating that YedW represses expression of the cyoABCDE operon. Taken together, we propose that YedW is a typical bifunctional regulator, acting as both an activator (hiuH) and a repressor (cyoABCDE).

**Fig. 6.** Cooperative regulation of the common target operons between YedWV and CusSR. After genomic SELEX screening, YedW-binding sites were identified for three intergenic spacer regions (cyoA<ampG, yedWV<hiuH and cusRS<=cusC). Using DNase I footprinting analysis, the YedW-binding sequence was identified as being the same as the CusR-binding sequence. Regulation in vivo by both YedW and CusR was confirmed using S1 nuclease and LacZ reporter assays. The product of hiuH (renamed from yedX) was identified as the enzyme catalysing the hydrolysis of 5-HIU (Hennebry et al., 2012).

**Cooperative regulation between YedWV and CusSR**

Here, we identified that YedW and CusR recognize and bind to the same sequence. If the functional forms of both TFs exist at high concentrations, they should compete with each other, but both belong to the low-abundance TF group in WT E. coli (Ishihama et al., 2014; Yamamoto et al., 2014). Under steady-state growth in the absence of external Cu²⁺ and H₂O₂, both should remain as non-phosphorylated non-functional forms. Thus, once E. coli is exposed to either Cu²⁺ or H₂O₂, activated CusR or YedW may participate in the activation of common targets. Furthermore, the activated forms of YedW and CusR might collaborate for enhanced expression of the same targets.

Copper is potentially toxic due in part to its ability to generate reactive oxygen species. Under aerobic conditions and in the presence of H₂O₂, the redox property of copper leads to the production of hydroxyl radicals via the Fenton reaction (Hodgkinson & Petris, 2012; Lemire et al., 2013). Hydroxyl radicals are reactive with most types of macromolecules, resulting in damage to lipids, proteins and nucleic acids. A second mechanism of copper toxicity is the destruction of protein structure through interactions with the polypeptide backbone or through binding of copper to amino acids, resulting in the exclusion of native metal cofactors. In particular, this process is damaging to iron–sulfur cluster proteins due to the high reactivity of sulfur to form thiolate bonds with Cu²⁺ (Keyer & Imlay, 1996; Macomber & Imlay, 2009). Thus, the simultaneous presence of two substrates, H₂O₂ and Cu²⁺, for the Fenton reaction should be highly toxic to cell growth, and thus the cooperative regulation of H₂O₂-sensing YedW and Cu²⁺-sensing CusSR is reasonable in order to express the genetic systems for survival in the presence of at least one of two stress signals.

The macrophage phagosome in host animals accumulates copper during bacterial infection, which may constitute an important killing mechanism of the infecting bacteria (Hodgkinson & Petris, 2012). The toxicity of copper is exploited to augment bacterial killing by the respiratory burst in the phagosome. The superoxide radical is short-lived and converts H₂O₂ non-enzymically. H₂O₂ produces highly reactive hydroxyl radicals via the Fenton reaction in the presence of metals such as Cu²⁺. However, bacteria express the genetic systems by expressing H₂O₂-sensing TFs including YedVVW and Cu²⁺-sensing TFs including CusSR.
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