

STUDIES ON THE SIGNAL TRANSDUCTION
NETWORK OF TWO COMPONENT SYSTEMS IN
ESCHERICHIA COLI

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STUDIES ON THE SIGNAL TRANSDUCTION NETWORK OF
TWO COMPONENT SYSTEMS IN *ESCHERICHIA COLI*

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SUMMARY

For survival, organisms adapt to environmental changes by switch the gene expression. Simple unicellular organisms that possess only thousands of genes such as bacteria shows the amazing adaptation ability against a variety of environment. Therefore, in bacteria, limited gene regulation mechanism may forms the transduction network with others for multiply the gene regulation pathway.

Two component system which is the major signal transduction pathway employed in wide varieties of bacteria, is generally composed of the sensor kinase (SK) that monitors an external signal(s) and the response regulator (RR) that controls physiological activities for response against external signals. A total of about 30 unique TCS pairs of SK and RR have been identified in *E. coli* based on the gene organization and the further genetic and/or biochemical data. In addition to these, there is the several RRs which pairing partner SK are not found and its function are unknown in *E. coli*. The process of TCS signal transduction generally show a high level of specificity, while a certain level of cross-regulation has been identified at the signal transduction pathways in *E. coli*: cross talk in recognition of signals by the sensor SK (stage 1); cross talk in phosphorylation of RRs by SKs (stage 2); and cross talk in recognition of regulation target promoters between RRs (stage 3). Cross talk between TCS pairs has been established at three stages of the signal transduction pathways.

Network formation between the TCSs may contribute for the bacterial adaptation to various environment. However, the perspective of the TCS network does not yet become clear. Especially, there are few reports for the cross recognition of the promoter by RRs. And *E. coli* possesses function unknown orphan RRs. In this study, for the elucidation of the entire signal transduction network of *E. coli*, I performed the comprehensive analysis of the stage 3 cross talk among RRs. And I investigated the role and activation mechanism of uncharacterized orphan RR.

The study of the stage 3 cross talk between NarL-family RRs is described in the chapter 2. In the same line study, the cross talk between OmpR family RRs were also analyzed and described in chapter 3. The chapter 4 focuses on the uncharacterized RR YgeK. Taking all the chapter together, my thesis presents the specific and complicated promoter recognition by RRs and function of atypical RR YgeK that plays the role of growth in acetate medium and biofilm formation. These findings provide the insight into the perspective of TCS signal transduction network and contribute for understanding the mechanism how bacteria adapt and survive against to environment change.

CHAPTER1

INTRODUCTION

1-1. Control of gene expression suitable for environment in bacteria

For survival, organisms adapt to environmental changes by switch the gene expression. Simple unicellular organisms that possess only thousands of genes such as bacteria shows the amazing adaptation ability against a variety of environment. That is, in bacteria, limited gene regulation mechanism may forms the transduction network with others for multiply the gene regulation pathway.

Escherichia coli (*E. coli*) contains a total of 4,453 protein-coding sequences on its genome. The selection of genes for transcription is determined by controlling the distribution of the limited number of RNA polymerase molecules on the genome (Ishihama, 2000; 2012). In *E. coli*, the promoter selectivity of RNA polymerase is determined after interaction with 7 species of the sigma subunit with the promoter recognition activity (Ishihama, 2000; 2012). The promoter selectivity of each holoenzyme is further modulated after interaction with a total of about 300 species of transcription factor (TF) (Ishihama, 2010; 2012; Yamamoto, 2014), of which about 10% are organized into the two-component system (TCS) (Mizuno, 1997; Yamamoto *et al.* 2005).

1-2. Two-component system (TCS)

TCS which is the major signal transduction pathway employed in wide varieties of bacteria (Egger *et al.* 1997), is generally composed of the sensor kinase (SK) that monitors an external signal(s) and the response regulator (RR) that controls physiological activities for response against external signals (Stock *et al.* 2000; Szurmant *et al.* 2007) (Fig. 1).

In the periplasmic region, SK senses extracellular or intracellular signals with amino-terminal sensory domain. Then, in the cytoplasmic region, carboxyl terminal CA (catalytic and ATP binding) domain phosphorylates conserved His residue on the DHp (dimerization and histidine phosphotransfer) domain. The His-bound phosphoryl group is then transferred onto a specific Asp residue on the cognate RRs. However, some histidine sensor kinases, known as hybrid sensor kinases, have a more complex type of phosphorelay consisting of two additional domains: a receiver domain containing a conserved Asp residue and a histidine-containing phosphotransmitter (HPt) domain. In such cases, signals are transmitted to RR through a His-Asp-His-Asp phosphorelay.

In the most of cases RR comprises a receiver domain and an effector domain whose activity is controlled by the phosphorylation state of the receiver. The receiver domain of RR consists of a five stranded parallel β -sheet surrounded by five amphipathic α -helices. Typically, the receiver domain possess the phosphate-accepting active pocket structure composed of the highly conserved Asp residue located at the end of β 3 strand, two other acidic amino acids (Asp/Glu) within the β 1- α 1 loop, an invariant Lys residue at the end of

the $\beta 5$ strand and a metal ion (usually Mg^{2+}) that are coordinated through hydrogen bonding (Gao & Stock, 2009). The phosphorylation of Asp residue commonly followed the reorientation of a highly conserved Thr/Ser residue at the end of $\beta 4$ strand and a highly conserved Phe/Tyr residue in the middle of $\beta 5$ strand toward the phosphorylated Asp residue which allows the formation of active dimer (Gao & Stock, 2009; Barbieri *et al.* 2013). The activated RRs in turn control not only transcription but also a variety of cellular processes including post-transcriptional or post-translational levels (Galperin, 2010). In a significant fraction of RRs, effector domains are enzymes that themselves participate in signal transduction: methyltransferases, adenylate or diguanylate cyclases, c-di-GMP-specific phosphodiesterases, histidine kinases, serine/threonine protein kinases and protein phosphatases.

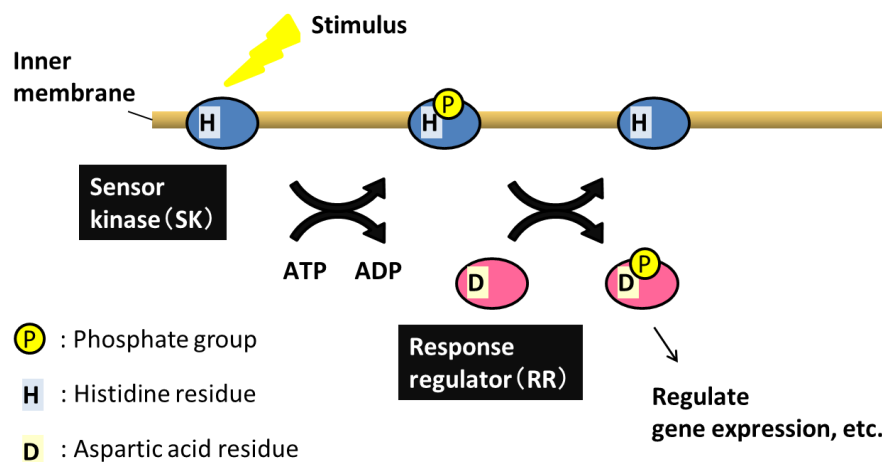


Fig. 1. Signal transduction of two-component system.

1-3. TCSs conserved in *Escherichia coli*

The genes encoding SK and RR pairs are typically organized adjacently on the *E. coli* genome, forming single operons, and thus the interacting SK-RR pair can be predicted based on the gene organization (Yamamoto *et al.* 2005) (Fig. 2). Although some SKs and RRs does not forms operon with cognate RRs or SKs, a total of about 30 unique TCS pairs of SK and RR have been identified in *E. coli* based on the further genetic and/or biochemical data (Laub & Goulian, 2007; Mizuno, 1997; Szurmant & Hoch, 2010; Yamamoto *et al.* 2005): ArcA-ArcB regulates the respiratory and fermentative metabolism in response to anaerobic condition (Gunsalus & Park, 1994; Malpica *et al.* 2006; Alvarez & Georgellis, 2010); BaeS-BaeR regulates the drug resistance and protein folding in response to envelop stress (Baranova & Nikaido, 2002; Nagakubo *et al.* 2002; Raffa & Raivio, 2002; Hirakawa *et al.* 2005; Nishino *et al.* 2005; Yamamoto *et al.* 2008); BasS-BasR prevent excessive Fe (III) binding in response to elevated levels of Fe (III) (Nagasawa *et al.* 1993; Chamnongpol *et al.* 2002; Yamamoto *et al.* 2005; Hagiwara *et al.* 2004); CpxA-CpxR regulates the various cell functions in response to envelop stress (Dorel *et al.* 2006; Snyder *et al.* 1995; Otto & Silhavy, 2002; Gupta *et al.* 1995; McEwen & Silverman, 1980; Lau-Wong *et al.* 2008; Dorel *et al.* 2006; Yamamoto & Ishihama, 2006); CreC-CreB regulates catabolic regulation in response to unknown catabolite (Avison *et al.*

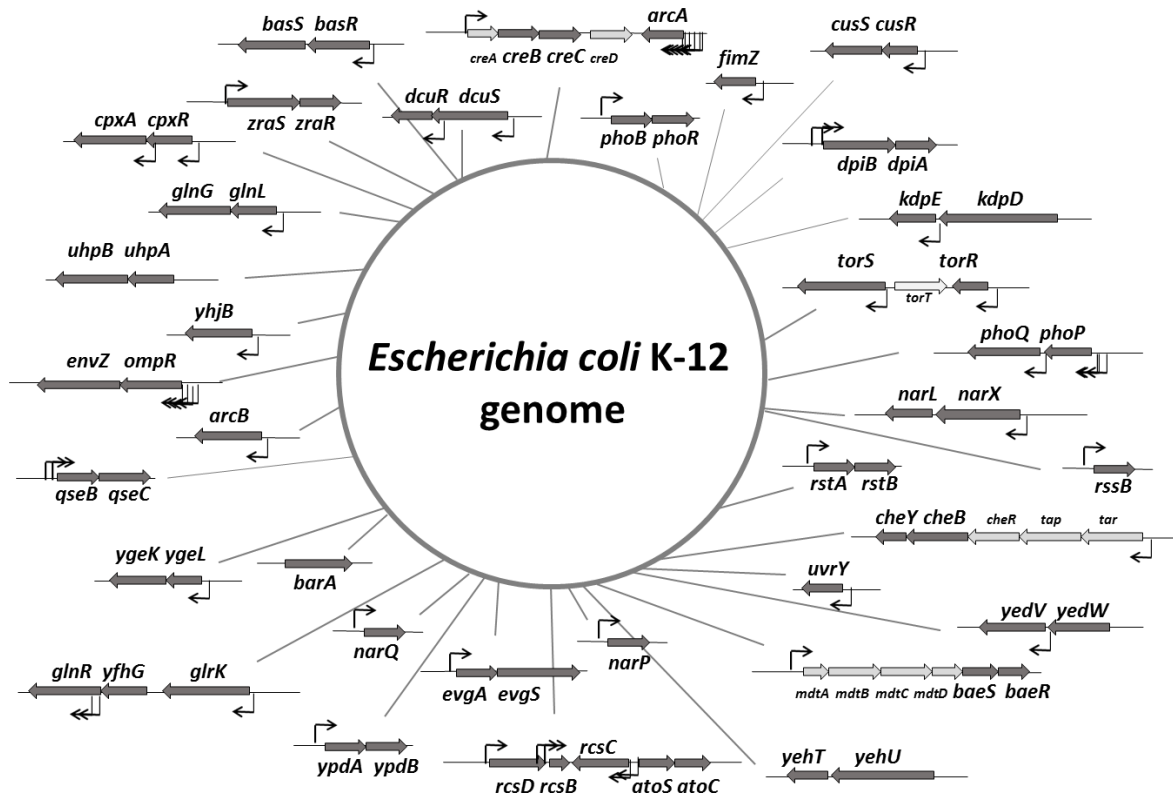


Fig. 2. Two-component systems conserved in *Escherichia coli* K-12 strain genome. Organization and position of TCS genes on the *E. coli* K-12 genome are shown.

2001; Kakuda *et al.* 1994; Duun *et al.* 1999; Richet 1996; Sprenger, 1995; Saveson & Lovett, 1999); CusS-CusR regulates the copper and silver efflux system in response to copper or silver ion (Outten *et al.* 2001; Munson *et al.* 2000; Franke *et al.* 2001); KdpA-KdpE regulates K⁺ uptake system in response to K⁺ limitation (Voelkner *et al.* 1993; Altendorf *et al.* 1994; Jung *et al.* 2000; Jung & Altendorf, 2002; Ballal *et al.* 2007); EnvZ-OmpR regulates outer membrane porin in response to osmotic stress (Garrett *et al.* 1985; Nara *et al.* 1986; Mizuno & Mizushima, 1987; Csonka & Hanson, 1991; Maeda *et al.* 1991; Kanamaru & Mizuno, 1992; Huang & Igo, 1996; Cai & Inouye, 2002; Yoshida *et al.* 2006); PhoR-PhoB regulates phosphorus uptake and metabolism in response to phosphate limitation (Wanner, 1993; VanBogelen *et al.* 1996; Baek & Lee, 2006); PhoQ-PhoP regulates Mg²⁺ homeostasis in response to Mg²⁺ limitation (Groisman *et al.* 1992; Kasahara *et al.* 1992; Kato *et al.* 1999); QseC-QseB (PmrB-PmrA) regulates flagella and motility in response to bacterial hormone (Sperandio *et al.* 2002); RstB-RstA regulates genes involved in various biological processes in response to low pH (Ogasawara *et al.* 2007; Yamamoto *et al.* 2005); TorS-TorR regulates TMAO induction in response to TMAO (Pascal *et al.* 1991; Simon *et al.* 1994); YedV-YedW regulates the copper and silver efflux systems in response to H₂O₂ (Urano *et al.* 2015); EvgS-EvgA regulates the acid and multidrug resistance in response to low pH and high concentration of alkali metal (Eguchi *et al.* 2003; Itou *et al.* 2009; Johnson *et al.* 2014); NarX-NarL regulates genes involved in anaerobic electron transport and fermentation in response to high-level nitrate and nitrite (Stewart 1994; Unden & Bongaerts 1997); NarQ-NarP also regulates genes involved in anaerobic electron transport and fermentation in response to low-level nitrate and

nitrite (Stewart 1994; Uden & Bongaerts 1997); RcsC-RcsD-RcsB regulates the biofilm formation and acid resistance in response to membrane perturbation (Huang *et al.* 2006; Clarke 2010); UhpB-UhpA regulates glucose hexose phosphate uptake in response to extracellular glucose hexose phosphate (Kadner & Shattuck-Eldens 1983; Weston & Kadner 1988); BarA-UvrY regulates the various cell processes such as carbon storage, biofilm formation, virulence and motility response for short-chain fatty acid (Pernestig *et al.* 2001); AtoS-AtoC regulates the metabolism of short-chain fatty acids, poly-(R)-3-hydroxybutyrate (cPHB) biosynthesis and flagellar synthesis in response to acetoacetate (Kyriakidis & Tiligada 2009); NtrB-NtrC (GlnL-GlnG) regulates the genes involved in the assimilation of nitrogen in response to nitrogen limitation (Burkovski 2003); GlnK-GlnR (YfhK-YfhA) upregulates *glmY* which is involved in synthesis of glucosamine-6-phosphate synthase when cells enter the stationary growth phase (Reichenbach *et al.* 2009); ZraS-ZraR (HydH-HydG) regulates zinc and lead efflux system response to high zinc or lead concentrate ions; CitA-CitB (DpiB-DpiA) regulates anaerobic citrate catabolism in response to citrate and anaerobic conditions (Bott 1997); DcuS-DcuR regulates C4-dicarboxylate metabolism in response to C4-dicarboxylates such as fumarate or succinate (Witan *et al.* 2012); YehU-YehT is considered that be involved in nutrient selection at the onset of stationary phase during growth in media containing peptides and amino acids as carbon source (Behr *et al.* 2014); YpdA-YpdB are also considered that be involved in nutrient selection at the onset of stationary phase response to extracellular pyruvate (Behr *et al.* 2014); CheA-CheY regulates the flagellar switch complex response to the changes in extracellular chemical concentrations (Lukat & Stock 1993).

On the other hand, pairing partner SK is not found for RR FimZ which function is unknown in *E. coli* and for RR RssB which involved in RpoS degradation. In addition to these, two orphan RR candidates YgeK and YhjB, both of these contain the conserved helix-turn-helix (HTH) motif of the RR family, were identified after detailed analysis of the *E. coli* K-12 genome sequence (Yamamoto *et al.* 2005). *In vitro* assay shows that RssB is phosphorylated by three SKs, ArcB, CheA and UhpB and YgeK is phosphorylated by two SKs, BarA and UhpB (Yamamoto *et al.* 2005).

1-4. RRs act as transcription factor in *E. coli*

Thirty-one of thirty-four RRs in *E. coli* is transcription factor containing HTH domain. These are classified to five family, OmpR (ArcA, BaeR, BasR, CpxR, CreB, CusR, KdpE, OmpR, PhoB, PhoP, QseB, RstA, TorR and YedW), NarL (EvgA, FimZ, NarL, NarP, RcsB, UhpA, UvrY, YgeK and YhjB), NtrC (AtoC, GlnR, NtrC and ZraR), CriR (CitB and DcuR) and LytT (YehT and YpdB), according to their HTH homology (Ishihama 2010, 2012; Yamamoto *et al.* 2005) (Table 1). Recognition DNA sequences of most of RRs had been reported up to the present. Those are very specific between RRs except for between NarL and NarP and between CusR and YedW which likely arose through gene duplication. However, recognition sequences are characteristic between RR families: OmpR-family RRs, exclude CusR and YedW, recognize directed repeat, NarL-family RRs recognize inverted repeat, NtrC-family RRs recognize inverted repeat, CriR-family RRs recognize directed repeat and LytT-family RRs recognize directed repeat.

Table 1. RR recognition sequence

Respose regulator		Recognition sequence	
ArcA	OmpR-family	Directed repeat	<u>GTTA ATTAAAT GTTA</u> Liu & De Wulf (2004)
BaeR	OmpR-family	Directed repeat	<u>TCTNCANAA (N)1 TCTNCANAA</u> Yamamoto <i>et al.</i> (2008)
BasR	OmpR-family	Directed repeat	<u>TTAANNNTT (N)1-2 TTAANNNTT</u> Ogasawara <i>et al.</i> (2012)
CpxR	OmpR-family	Directed repeat	<u>GTAAA (N) 4-8 GTAAA</u> Yamamoto <i>et al.</i> (2006)
CreB	OmpR-family	Directed repeat	<u>TTCAC (N)6 TTCAC</u> Cariss <i>et al.</i> (2008)
CusR	OmpR-family	Inverted repeat	<u>AAAATGACAA (T/A) (T/A) TTGTCATTTT</u> Yamamoto <i>et al.</i> (2005)
KdpE	OmpR-family	Directed repeat	<u>TTTTTA(T/C)AC (N)2 TTTTTA(T/C)AC</u> Sugiura <i>et al.</i> (1992)
OmpR	OmpR-family	Directed repeat	<u>TTTACTTTTT GNAACATNTT</u> Harlocker <i>et al.</i> (1995)
PhoB	OmpR-family	Directed repeat	<u>CTGTCATA(T/A)A (T/A) CTGTCACA(T/A)(T/A)</u> Blanco <i>et al.</i> (2002)
PhoP	OmpR-family	Directed repeat	<u>(T/G)(G/A)TT(T/G)A (N)5 (T/G)(G/A)TT(T/G)A</u> Yamamoto <i>et al.</i> (2002)
QseB	OmpR-family	Directed repeat	<u>A(A/T)T(A/T) (N)2 A(A/T)T(A/T)</u> Clarke & Sperandio (2005)
RstA	OmpR-family	Directed repeat	<u>TACA TNTNGT TACA</u> Ogasawara <i>et al.</i> (2007)
TorR	OmpR-family	Directed repeat	<u>CTGTTCAT (N)2-14 CTGTTCAT</u> Simon <i>et al.</i> (1995)
YedW	OmpR-family	Inverted repeat	<u>AAAATGACAA (T/A)(T/A) TTGTCATTTT</u> Urano <i>et al.</i> (2015)
EvgA	NarL-family	Inverted repeat	<u>TTC(C/T)TACA (N)2 TGTA(A/G)GAA</u> Masuda & Chruch (2003)
FimZ	NarL-family	Unknown	Unknown -
NarL	NarL-family	Inverted repeat	<u>TAC(C/T)(C/T)(A/C)T (N)2 A(G/T)(A/G)(A/G)GTA</u> Stewart & Bledsoe (2003)
NarP	NarL-family	Inverted repeat	<u>TAC(C/T)(C/T)(A/C)T (N)2 A(G/T)(A/G)(A/G)GTA</u> Stewart & Bledsoe (2003)
RcsB	NarL-family	Inverted repeat	<u>TAGGATT AATCCTA</u> Yamamoto <i>et al.</i> (Unpublished)
UhpA	NarL-family	Inverted repeat	<u>(A/G)CCTG(A/G)(A/G) (N)6 (C/T)(C/T)CAGG(C/T)</u> Olekhovich & Kadner (2002)
UvrY	NarL-family	Inverted repeat	<u>TGTAAGAGA TCTCTTACA</u> Martínez <i>et al.</i> (2014)
YgeK	NarL-family	Unknown	Unknown -
YhjB	NarL-family	Unknown	Unknown -
AtoC	NtrC-family	Inverted repeat	<u>G(G/T)TAT(C/A)(G/C)ATCCG N</u> <u>CGGAT(G/C)(T/G)ATA(A/C)C</u> Matta <i>et al.</i> (2007)
GlrR	NtrC-family	Inverted repeat	<u>TGTC (N)10 GACA</u> Reichenbach <i>et al.</i> (2009)
NtrC	NtrC-family	Inverted repeat	<u>TGCACC (N)5 GGTGCA</u> Porter <i>et al.</i> (1993)
ZraR	NtrC-family	Inverted repeat	<u>GAGTAAAAATGACTCGC (N)12</u> <u>GCGAGTCATTTTACT</u> Leomhartsberger <i>et al.</i> (2001)
CitB	CriR-family	Directed repeat	<u>TTTA (N)4-5 TTTA</u> Yamamoto <i>et al.</i> (2009)
DcuR	CriR-family	Directed repeat	<u>(T/A)(A/T)(T/C)(A/T)AA (N)1-4</u> <u>(T/A)(A/T)(T/C)(A/T)AA</u> Abo-Amer <i>et al.</i> (2004)
YehT	LytT-family	Directed repeat	<u>ACC(G/A)CT(C/T)A (N)13 ACC(G/A)CT(C/T)A</u> Kraxenberger <i>et al.</i> (2012)
YpdB	LytT-family	Directed repeat	<u>GGCATTTTCAT N(11) GGCATTTTCAT</u> Fried <i>et al.</i> (2012)

1-5. Cross talk of bacterial two-component systems

Signal transduction network in eukaryotes display extensive cross talk with individual kinase acting on large number of targets (Ptacek *et al.* 2005). On the other hand, in bacteria, the process of TCS signal transduction generally show a high level of specificity; HK usually act on a single target (Laub & Gourian, 2007; Capra & Laub, 2012). But a certain level of cross-regulation has been identified at the signal transduction pathways in *E. coli* : cross talk in recognition of signals by the sensor SK (stage 1); cross talk in phosphorylation of RRs by SKs (stage 2); and cross talk in recognition of regulation target promoters between RRs (stage 3). Cross talk between TCS pairs has been established at three stages of the signal transduction pathways (Fig. 3).

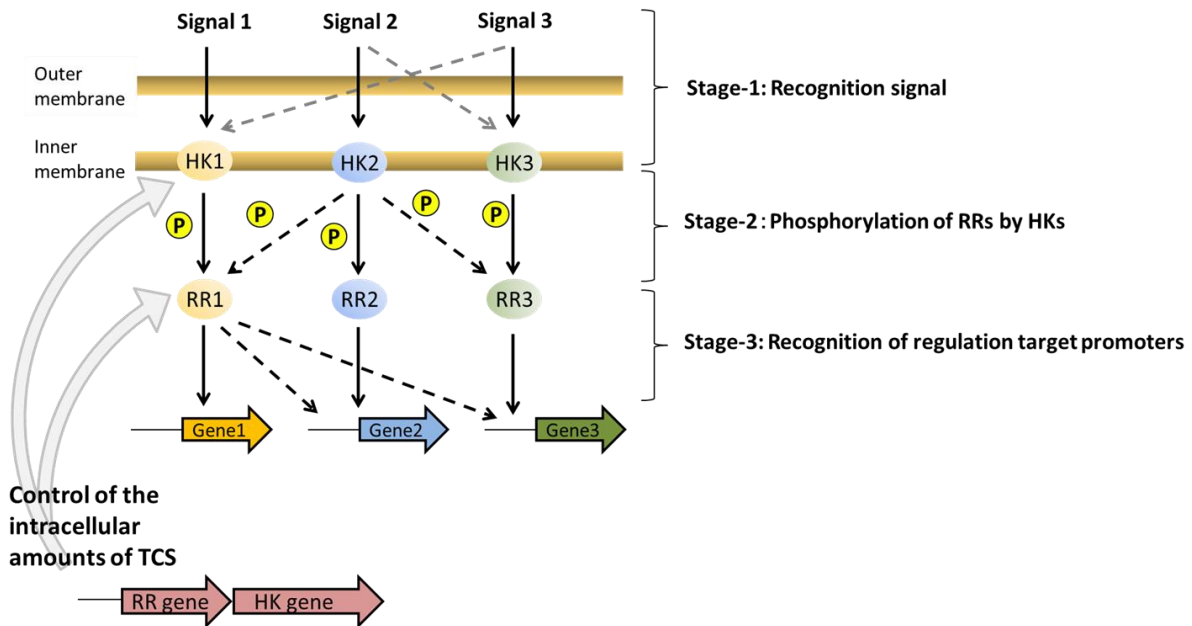


Fig. 3. Overview of signal transduction by two-component systems.

As to the stage 1, one well-characterized cross talk is the recognition of metals with metal-sensing SKs (Yamamoto 2014). Besides highly reactive soft metals such as Na and K, bacteria require trace amounts of some transition metals, heavy metals, and metalloids, but these metals are usually toxic in excess. In *E. coli*, the intracellular level of metal is controlled by the export of excess and/or the transport of limit metal ion (Hobman *et al.* 2007; Yamamoto 2014). In *E. coli*, a total of 13 species of the metal-specific regulators have been identified, which are involved in recognition of metals in environment and in transcription regulation of the genes encoding the cellular systems for metal homeostasis (Yamamoto 2014). The metal elements (Na, Mn, Ni, Cu, Zn, Fe, and Mo) are directly recognized by the DNA-binding TFs (NhaR, MntR, NikR, CueR, ZntR/Zur, Fur, and ModE, respectively). Other metals (K, Mg, Cu, Zn, and Fe) are recognized by TCSs (KdpDE, PhoQP, CusSR, ZraSR, and BasSR, respectively), but between these metal-sensing SKs, high levels of the cross-recognition of noncognate metals have been identified (Hobman *et al.* 2007; Yamamoto 2014).

As to the cross talk at the stage 2 of signal transduction, a systematic and comprehensive analysis of the activity and specificity of self-phosphorylation *in vitro* of SK and transphosphorylation *in vitro* of RR by phosphorylated SK for all possible combinations between SKs and RRs purified from over-expressed *E. coli* had been carried out. Result of these experiment, cross talks in *E. coli* TCS was observed for the 21 (3.0%) combination out of a total of 692 non-cognate pairs examined (Yamamoto *et al.* 2005). For the *in vivo* assay, cross talk at the phosphotransfer also have been reported in *E. coli*: NarX and NarQ cross-phosphorylate NarL and NarP (Tyson *et al.* 1994); CreC phosphorylate PhoB in the absence of PhoR (Kim *et al.* 1996); PhoB phosphorylated by acetylphosphate via EnvZ in the absence of PhoR (Kim *et al.* 1996); ArcB phosphorylate OmpR under the anaerobic condition (Matsubara *et al.* 2000); CpxA phosphorylate OmpR in the absence of EnvZ and CpxR, vice versa EnvZ phosphorylate CpxR in the absence of CpxA and OmpR (Siriyaporn &

Goulian, 2008); PmrB (BasS) phosphorylates QseB in response to Fe³⁺ in the absence of QseC (Guckes *et al.* 2013). When one RR is phosphorylated by multiple species of SK, the target genes under the control of this particular RR become responsible to various stresses. Likewise, some SKs are able to phosphorylate multiple RRs, ultimately resulting in the expansion of regulation targets. The HK–RR interactions underlying this stage 2 cross talk may provide an efficient starting point for the evolution of new signaling circuits for adaptation under varieties of environment.

The stage 3 cross talk involves a target gene promoter with multiple RR-recognition sequences. Considering the specificity of recognition sequences, most RRs are expected to bind to different positions in the promoter region, whilst limited sets of RRs, including NarL/NarP and CusR/YedW (Darwin *et al.* 1997; Urano *et al.* 2015), can recognize the same nucleotide sequence. In spite of such strict specificity, recognition sequences of different RRs often shares a common motif. Subtle differences in the recognition sequences may prevent RRs from unnecessary cross-regulation. For instance, PhoB also binds to the CusR and YedW binding position on the *cusR* and *cusC* promoter responds to phosphate limitation although consensus sequence of PhoB and CusR/YedW are different (Yang *et al.* 2012) (Table 1). On the *ompF* promoter, four CpxR and four OmpR binding site are present and only three of those site is recognized by CpxR and OmpR to overlap each other. On the *csgD* promoter, has twelve CpxR binding site, BasR recognizes the one of CpxR binding site and OmpR/RstA recognize the other one to overlap with CpxR (Batchelor *et al.* 2005; Yoshida *et al.* 2006; Ogasawara *et al.* 2010), although consensus sequences of CpxR, OmpR, RstA and BasR are diffent (Table 1). In addition to these, according to the charactristics of recognition sequences between RR families, directed repeat or inverted repeat, cross-recognition may happen between RRs belongs to same family. In any case, overview of cross talk at the stage 3 has not been revealed. Therefore it has not been understood that how frequency RRs bind to same sequence.

1-6. Object of this study

Formation of signal transduction network among the TCSs is considered that contribute for the bacterial adaptation ability for various environment. However, the perspective of the TCS network does not yet become clear. Especially, there are few reports for the cross recognition of the same sequence by RRs. In this study, for the elucidation of the entire signal transduction network of *E. coli*, I performed comprehensive analysis of the stage 3 cross talk between RRs belongs to same family. In addition to this, *E. coli* possess function unknown RRs. Therefore, I also analyzed the function and activation mechanism of function unknown RR.

CHAPTER2

CROSS TALK IN PROMOTER RECOGNITION BETWEEN SIX-NARL FAMILY RESPONSE REGULATORS OF *ESCHERICHIA COLI* TWO-COMPONENT SYSTEM

2-1. Introduction

NarL and NarP which are belong to NarL-family with similar HTH domain had been known that recognize the same sequence. Recent study shows that CusR and YedW which are belong to OmpR-family, these are also possess very similar DNA binding domain, recognize the same sequence (Urano *et al.* 2015). In addition to these cross recognition, there are report that OmpR-family RR PhoB also bind to CusR and YedW binding position (Yang *et al.* 2012) and that OmpR-family RR BasR and CpxR or CpxR, OmpR and RstA bind to same sequence to overlap each other. To determine whether such a thing occurs between other similar RRs or not and how much frequency dose it occurs, I analyzed in this study the stage 3 cross talk in the recognition of promoters between different RRs belong to same family.

As a model system, I analyzed the cross-regulation between six members of the NarL-family RRs: EvgA (*E.coli* homologous of *Bordetella* BvgA: *Bordetella* virulence gene; regulator for acid resistance), NarL (nitrate/nitrite response regulator L), NarP (nitrate/nitrite response regulator P), RcsB (regulator capsule synthesis B), UhpA (regulator for uptake of hexose phosphates), and UvrY (ultraviolet response regulator). For each of these six NarL-family RRs, a representative promoter were selected and the recognition *in vitro* and *in vivo* of noncognate promoters were examined.

2-2. Materials and Methods

2-2-1. *E. coli* strains and growth conditions

Escherichia coli strains used in this study are summarized in Table 1. *E. coli* W3110 type-A was used for preparation of the expression clones of RRs, and of the regulation target promoters. *E. coli* BL21 (DE3) was used for expression and purification of RRs. *E. coli* BW25113 was used for the reporter assays of promoters under the control of test TFs. Cells were cultured in LB medium or M9-0.4% glycerol medium at 37°C. When necessary, 100 µg/mL ampicillin, 25 µg/mL kanamycin, or 20 µg/mL chloramphenicol was added into the medium. Cell growth was monitored by measuring the optical density at 600 nm.

2-2-2. Construction of arabinose-inducible expression system of RRs

The coding sequences of six species of NarL-family RR (EvgA, NarL, NarP, RcsB, UhpA, and UvrY) were PCR amplified using 5'-proximal and 3'-proximal primers of each open reading frame (for the sequence, see Table 2). Into all the 5'-primer sequence, the typical ribosome recognition sequence (SD sequence) was added. In addition, all the 3'-primers included a FLAG-tag sequence so as to be expressed as fusion with RRs. The PCR-amplified FLAG-tagged RR-coding sequences were inserted into *Eco*RI and *Hind*III treated pBAD33 to construct the arabinose-inducible expression plasmids of FLAG-tagged RRs by In-Fusion HD (Clontech). The plasmid construct was confirmed by DNA sequencing (Table 3).

Table 1. Bacterial strains, bacteriophage

Bacterial strains		
<i>Escherichia coli</i> W3110 type-A	F ⁺ IN(<i>rrnD-rrnE</i>) <i>rph-1</i>	Jishage & Ishihama (1996)
<i>Escherichia coli</i> DH5α	F ⁺ <i>endA1 supE44 thiE1 recA1 gyrA96 deoR481 phoA8</i>	
<i>φ80ΔlacZ(M15) ΔhsdR17 (rK- mK+) Δ(argF-lac)169</i>		
<i>Escherichia coli</i> MC4100	F ⁺ <i>araD139 Δ(argF-lac)169 flhD5301 thiA1 relA1 rpsL150</i>	
<i>ptsF25 rbsR22 deoC1Δ(fimB-fimE)</i>	Casadaban (1976)	
<i>Escherichia coli</i> BW25113	F ⁺ <i>rrnB3ΔlacZ4787 hsdR514Δ(arabAD)567Δ(rhaBAD)568 rph-1</i>	Datsenko <i>et al.</i> (2000)
<i>Escherichia coli</i> MY0901	BW25113 <i>λydeP-lacZ</i>	This study
<i>Escherichia coli</i> MY06131	BW25113 <i>λnirB-lacZ</i>	This study
<i>Escherichia coli</i> MY06133	BW25113 <i>λuhpT-lacZ</i>	This study
<i>Escherichia coli</i> MY06134	BW25113 <i>λwza-lacZ</i>	This study
<i>Escherichia coli</i> MY0702	BW25113 <i>λcsrB-lacZ</i>	This study
Bacteriophages		
λRS45	<i>bla-lacZ imm21 ind</i>	Simons <i>et al.</i> (1987)

2-2-3. Expression and purification of His-tagged RR proteins

Expression of His-tagged RR proteins was carried out essentially according to the standard procedure (Yamamoto *et al.* 2005; Ishihama *et al.* 2014). For construction of plasmid for expression of His-tagged RRs, the RR-coding sequences were amplified by PCR using *E. coli* W3110 genome DNA as a template and a pair of primers, which were designed so as to hybridize upstream or downstream of the RR-coding sequence (for the sequence, see Table 2). After digestion with *Bam*HI and *Not*I (note that the restriction enzyme sites were included within the primer sequences), PCR products were cloned into pET21a (+) (Novagen) between *Bam*HI and *Not*I sites. The plasmid construct was confirmed by DNA sequencing (Table 3). Purification of His-tagged RRs was carried out essentially according to Yamamoto *et al.* (2005).

2-2-4. Gel mobility shift assay

Gel mobility shift assay was carried out as described previously (Nakano *et al.* 2014; Yamanaka *et al.* 2014). A set of 5'-fluorescein isothiocyanate (FITC)-labeled primers were prepared as described in Table S1 (Supporting Information). For gel shift assays, each of the FITC-labeled probes was incubated at 37°C for 30 min with various amounts of protein in a transcription buffer consisting of 50 mM Tris-HCl, pH 7.8 at 4°C, 3 mM Mg acetate, 0.1 mM EDTA, 0.1 mM DTT, 0.025 mg/mL BSA and 50 mM NaCl and 10 mM acetyl phosphate. Sonicated and denatured salmon testes DNA (Nacalai) was added to absorb nonspecific DNA-binding proteins. After the addition of a DNA dye solution, the mixture was directly subjected to 6% PAGE. Fluorescent-labeled DNA in gels was detected using LAS-4000 CCD camera (Fujifilm).

2-2-5. Zn²⁺Phos-tag SDS-PAGE

Zn²⁺Phos-tag SDS-PAGE was carried out as described previously (Kinoshita *et al.* 2014). The separating gel is consisted of 10% w/v polyacrylamide and 357mM 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)

Table 2. Oligonucleotide**Used for reporter plasmid**

YDEP-P-LF	GGGTGGAATTCTAATGCGTAATGGAAAAAT	This study
YDEP-P-LR	GCCATGGATCCCCGGACAGTCAAAGCCCT	This study
NIRB-P-LF	TACCAGAATTCTCACCTGGGTTCACAGCA	This study
NIRB-P-LR	GAGAGGGATCCGACGAGAGGTGTACGCGGT	This study
WZA-P-LF	AATAAGAATTCATTTAAGAAATATCGCATG	This study
WZA-P-LR	GCGGAGGATCCTTCACCATTTTGTGAGAT	This study
UHPT-P-LF	GCAACGAATTCCTGATGAATTTGATTTTCG	This study
UHPT-P-LR	CGTTCGGATCCATGTTAAAGTTCTTGCGAA	This study
CSRB-P-LF	GACTCGAATTCCTGGGGATATGCACGCGCA	This study
CSRB-P-LR	GTGTGGGATCCAGAAGTGTATCATCCTGA	This study
UHPT_LR_F1	TCGTCTTCACCTCGACTTTTGAACGCCAGACACCGCGC	This study
UHPT_LR_R1	ACTAACTAGAGGATCAGCCAGCATGGGTTACTCCTGAAAT	This study

Used for RR expression plasmid

EVGAF-1	TTGGGCTAGCGAATTAGGAGGAATTCACCATGAACGCAAT AATTATTGA	This study
EVGAR-1	TACCGAGCTCGAATTTTACTATTTATCGTCGTCATCTTTGTA GTCGCCGATTTTGTACGTTGTG	This study
NARLF-1	TTGGGCTAGCGAATTAGGAGGAATTCACCATGAGTAATCA GGAACCGGC	This study
NARLR-1	TACCGAGCTCGAATTTTACTATTTATCGTCGTCATCTTTGTA GTCGAAAATGCGCTCCTGATGCA	This study
NARPF-1	TTGGGCTAGCGAATTAGGAGGAATTCACCATGCCTGAAGC AACACCTTTTCA	This study
NARPR-1	TACCGAGCTCGAATTTTACTATTTATCGTCGTCATCTTTGTA GTCTTGTGCCCGCGTTGTTGCA	This study
RCSBF-1	TTGGGCTAGCGAATTAGGAGGAATTCACCATGAACAATATG AACGTAATTAT	This study
RCSBR-1	TACCGAGCTCGAATTTTACTATTTATCGTCGTCATCTTTGTA GTCGTCTTTATCTGCCGGACTTA	This study
UHPAF-1	TTGGGCTAGCGAATTAGGAGGAATTCACCATGATCACCGT TGCCCTTATAGA	This study
UHPAR-1	TACCGAGCTCGAATTTTACTATTTATCGTCGTCATCTTTGTA GTCCCAGCCATCAAACATGCGGC	This study
UVRYF-1	TTGGGCTAGCGAATTAGGAGGAATTCACCATGATCAACGT TCTACTTGTTGA	This study
UVRYR-1	TACCGAGCTCGAATTTTACTATTTATCGTCGTCATCTTTGTA GTCCTGACTTGATAATGTCTCCG	This study

Used for Protein purification

EVGAF	CAAAGGGAAGGATCCATGAACGCAATAATT	Yamamoto, K <i>et al.</i> 2005
EVGAR	AAAAACTTCAGCGGCCGCGCCGATTTTGT	Yamamoto, K <i>et al.</i> 2005
NARLF	TCCAAGGAGGGATCCATGAGTAATCAGGAA	Yamamoto, K <i>et al.</i> 2005
NARLR	GCTGGGAACGCGGCCGCGAAAATGCGCTCC	Yamamoto, K <i>et al.</i> 2005
NARPF	CCTCAGGAGGGATCCATGCCTGAAGCAACA	Yamamoto, K <i>et al.</i> 2005
NARPR	CCATCGGGCGCGGCCGCTTGTGCCCGCGT	Yamamoto, K <i>et al.</i> 2005
RSCBF	CAAGGTAGCCGGATCCATGAACAATATGAA	Yamamoto, K <i>et al.</i> 2005
RSCBR	TATCTGGCCTACAGCGGCCGCGTCTTTATC	Yamamoto, K <i>et al.</i> 2005
UHPAF	CGCCAGGACGGATCCATGATCACCGTTGCC	Yamamoto, K <i>et al.</i> 2005
UHPAR	GAACAACGTGCGGCCGCCAGCCATCAAAC	Yamamoto, K <i>et al.</i> 2005
UVRYF	ATTTCTGGAGATGGATCCTTGATCAACGTT	Yamamoto, K <i>et al.</i> 2005
UVRYR	CGTCAAACGTGGCGGCCGCTGACTTGATAA	Yamamoto, K <i>et al.</i> 2005

Used for Gel mobility shift assay

lac30R	GCTGCAAGGCGATTAAGTT	Kurata, T <i>et al.</i> 2013
FITC_lac	AGGGTTTTCACGATCACGACGTTGTAAAAC	Kurata, T <i>et al.</i> 2013

Table 3. Plasmids

pRS552	promoter-less <i>lacZ</i> , Ap ^r	Simons <i>et al.</i> (1987)
pBAD33	pACYC184 derived, P _{BAD} Cm ^r	Guzman <i>et al.</i> (1995)
pKH57-21	pET21a(+) (<i>evgA</i>)	Yamamoto <i>et al.</i> (2005)
pKH10-2	pET21a(+) (<i>narL</i>)	Yamamoto <i>et al.</i> (2005)
pKH26-2	pET21a(+) (<i>narP</i>)	Yamamoto <i>et al.</i> (2005)
pKH28-1	pET21a(+) (<i>rcsB</i>)	Yamamoto <i>et al.</i> (2005)
pKH46-3	pET21a(+) (<i>uhpA</i>)	Yamamoto <i>et al.</i> (2005)
pKH18-2	pET21a(+) (<i>uvrY</i>)	Yamamoto <i>et al.</i> (2005)
pYDEPP	pRS552 (<i>ydeP-lacZ</i>)	This study
pNIRB	pRS552 (<i>nirB-lacZ</i>)	This study
pWZA	pRS522 (<i>wza-lacZ</i>)	This study
pUHPT	pRS522 (<i>uhpT-lacZ</i>)	This study
pCSRB	pRS522 (<i>csrB-lacZ</i>)	This study
puhpT-luxL1	pLUX (<i>uhpT-lux</i>)	This study
pBADEvgA-FLAG	pBAD33, FLAG-tagged EvgA at C-terminus	This study
pBADNarL-FLAG	pBAD33, FLAG-tagged NarL at C-terminus	This study
pBADNarP-FLAG	pBAD33, FLAG-tagged NarP at C-terminus	This study
pBADRcsB-FLAG	pBAD33, FLAG-tagged RcsB at C-terminus	This study
pBADUhpA-FLAG	pBAD33, FLAG-tagged UhpA at C-terminus	This study
pBADUvrY-FLAG	pBAD33, FLAG-tagged UvrY at C-terminus	This study

2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol-HCl (pH 6.8). 0.1 mM Phos-tag Acrylamide and 0.2 mM of ZnCl₂ were added to the separating gel before polymerization. An acrylamide stock solution was prepared containing a 29:1 mixture of acrylamide to *N*', *N*'-methylenebisacrylamide. The running buffer is consisted of 0.1M Tris and 0.1M MOPS containing 0.10% w/v SDS and 5.0 mM NaHSO₃. The NaHSO₃ was dissolved immediately before use. Electrophoresis was performed at 30mA/gel at 4°C until the BPB dye reached the bottom of the separating gel.

2-2-6. Western blot analysis

Expression and purification of His-tagged RRs were determined by Western blot system (Jishage & Ishihama, 1996) using anti-RR antibodies that were raised in rabbits using purified RRs, whereas the expression level of FLAG-tagged RRs was determined by Western blot analysis using anti-FLAG antibody. After SDS-PAGE, proteins were transferred onto PVDF membrane using iBlot gel transfer system (Invitrogen). Membranes were washed with skim milk in TBS buffer and then treated with anti-RR or anti-FLAG antibodies. The antibodies bound were detected using HRP (horseradish peroxidase)-linked anti-mouse IgG antibody as the secondary antibody. The chemiluminescence was measured using LAS-4000 CCD camera (Fujifilm).

2-2-7. Reporter assay: LacZ system

For detection of the regulatory roles of six test RRs, a set of reporter assay strains were constructed. In brief, approximately 650-bp-long sequence between -500 and +150 with respect to the initiation codon of each regulation target gene was PCR-amplified using a pair of the primers (for the primer sequences, see

Table 2). After digestion with *EcoRI* and *BamHI*, the promoter fragment was inserted into *EcoRI*- and *BamHI*-treated pRS552 (Simons *et al.* 1987), leading to construct a set of promoter assay vector (Table 3). For construction of the single-copy LacZ vector, these plasmids were transformed into *E. coli* MC4100, into which λ RS45 was infected. Resultant lysogenic phages were transfected into *E. coli* BW25113, and their lysogens, each carrying a single copy of test promoter-*lacZ* fusion, were used for the LacZ reporter assay. The assay of β -galactosidase activity was carried out using the standard procedure (Simons *et al.* 1987). In brief, an aliquot of cell culture was mixed with a reaction mixture (Z-buffer plus SDS and chloroform) and then treated with ONPG (Yamamoto *et al.* 2011). The yellow color developed from ONPG was measured at 420 nm. The activity was expressed as the Miller unit.

2-2-8. Reporter assay: Lux system

All five RR-dependent promoters-lux (luciferase) fusions were inserted into pLUX vector (Table 3) (Blouin *et al.* 1996; Burton *et al.* 2010). The construction of resulting plasmids was confirmed by DNA sequencing. Transformants carrying one of these plasmids were grown at 37°C with shaking in LB medium. At the middle of exponential phase, the culture was transferred to a microtiter plate (96-well microtiter) to start monitoring the luciferase activity with an automated plate reader MTP-880 (Corona).

2-3. Results

2-3-1. Test systems of the stage 3 cross talk of *E. coli* NarL-family RRs

As an initial attempt of search for the cross talk at the stage 3 of TCS signal transduction, an attempt was made to examine the promoter recognition properties of six NarL-family RRs: NarL, NarP, EvgA, UhpA, RcsB, and UvrY (Table 4-A). NarL and NarP are transcriptional regulators of a number of anaerobic electron transport and fermentation-related genes (Stewart 1994; Uden & Bongaerts 1997). NarL functions as the superior activator at high concentrations of nitrite, whereas NarP plays an activation role at low nitrite concentration. EvgA is activated by EvgS SK cascade and regulates the genes involved in acid resistance and multidrug resistance (Eguchi *et al.* 2003; Itou *et al.* 2009; Johnson *et al.* 2014). RcsB RR is activated by the RcsC/RcsD cascade (Huang *et al.* 2006; Clarke 2010) and controls colanic acid production for biofilm formation (Stout 1994). UhpA also forms a three-component system with UhpB SK and UhpC accessory protein (Kadner & Shattuck-Eldens 1983; Weston & Kadner 1988). UvrY was considered to be an orphan RR, but later found to be phosphorylated for activation by BarA SK (Pernestig *et al.* 2001), which senses short-chain fatty acid. As a model promoter for each of these six RRs, I selected one representative gene from a set of the genes that have so far been identified as the regulation targets by each RR: *ydeP* for EvgA (Masuda & Church 2003; Johnson *et al.* 2014); *nirB* for NarL and NarP (Wang & Gunsalus 2000); *uhpT* for UhpA (Ambudkar *et al.* 1986; Weston & Kadner 1988); *wza* for RcsB (Drummelsmith & Whitfield 1999); and *csrB* for UvrY (Liu *et al.* 1997; Suzuki *et al.* 2002) (Table 4-B).

2-3-2. Activation and controlled expression of six NarL-family RRs

Previously, the intracellular concentrations and the expression levels of TFs at various phases of *E. coli* growth are both determined (Ishihama *et al.* 2014; Yamamoto *et al.* 2014). The levels are different between the RRs in both exponential growth and stationary phases. I then set up two different culture systems for control of the intracellular level of test RRs: (i) enhancement of the intracellular level and/or activity of RRs upon exposure to the hitherto identified induction conditions; and (ii) artificial over-expression of the test RRs using the arabinose-dependent protein expression vector. The level of activated RR was examined using the reporter systems. Activation of EvgA could be achieved upon exposure to an acidic condition (Eguchi & Utsumi 2014), whereas UhpA was activated after the addition of inducer glucose-6-phosphate (Weston & Kadner 1988). For other RRs, however, high-level activation was not observed using the known induction conditions: the addition of nitrate for NarL and NarP (Constantindou *et al.* 2006); the addition of ZnCl₂ at 20°C for RcsB (Hagiwara *et al.* 2003); and the addition of formate at pH5.0 for UvrY (Chavez *et al.* 2010). In these cases, as yet unidentified factors or conditions might be needed for activation. Then, a controlled expression system for the test RR proteins were established. For detection of the expression level, the RR genes were tagged with FLAG-tag sequence at 3'-terminus and then inserted into an arabinose-inducible expression vector. The arabinose concentration-dependent expression of FLAG-tagged RRs was confirmed by immunoblotting with use of anti-FLAG antibody (Fig. 1-A). The maximum level of expression was observed for all test RRs after the addition of arabinose as low as 0.002%. For all of RRs, induction of cognate promoter expression was achieved in this condition. For EvgA and UhpA, however, those were more activated upon exposure to the hitherto identified induction conditions than RR artificial over-expression.

Using the best condition to give the highest promoter activity for each RR, the cross-recognition of

Table 4. NarL-family RRs and their Promoters

A) Test response regulator

SK	RR	Regulatory roles	Regulation target operons
EvgS	EvgA	Regulator for acid and multidrug resistance	<i>gadE-mdtEF, acrD, evgAS, emrKV, ydeP, safA-ydeO</i> (9)
NarX	NarL	Regulator against high-level nitrate and nitrite	<i>citCDEFXG, dpiBA, nirBDC, hyaABCDEF, narGHJI</i> (33)
NarQ	NarP	Regulator against low-level nitrate and nitrite	<i>hyaABCDEF, napFDAGHEC, nirBDC, nrfABCDEFG</i> (11)
RcsCD	RcsB	Regulator of colanic acid in biofilm formation	<i>leuO, bglGFB, yidL, sfsB, ynbABCD, yecT, ykiA</i> (35)
UhpBC	UhpA	Regulator of hexose phosphate uptake	<i>uhpT</i> (1)
BarA	UvrY	Regulator of carbon storage system	<i>csrB, csrC</i> (2)

B) Test promoter

RR	Test promoter	Gene product	Protein function
EvgA	<i>ydeP</i>	AraC family transcription factor	regulation of acid resistance genes
NarL	<i>nirB</i>	nitrite reductase enzyme	reduction of nitrite to ammonia
NarP	<i>nirB</i>	nitrite reductase enzyme	reduction of nitrite to ammonia
RcsB	<i>wza</i>	outer membrane lipoprotein	capsular polysaccharide translocation
UhpA	<i>uhpT</i>	Major Facilitator Superfamily (MFS) transporter	hexose-6-phosphate:phosphate antiporter
UvrY	<i>csrB</i>	small regulatory RNA (sRNA)	regulatory sRNA for carbon storage

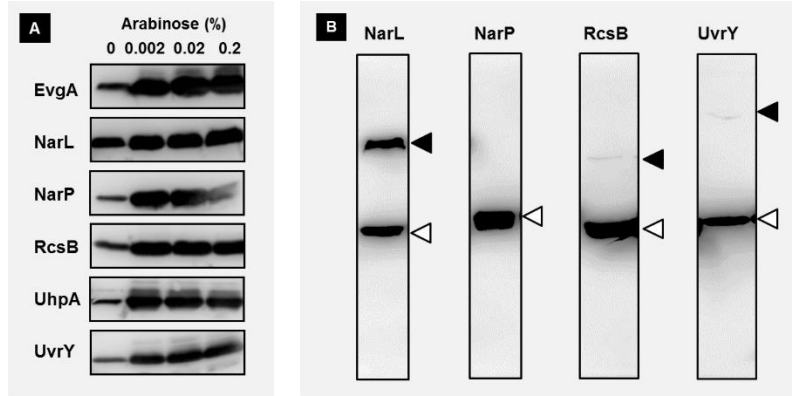


Fig. 1. Artificial expression of RRs. The coding sequences of six NarL-family RRs with FLAG-tag sequence at 3'-termini were inserted into the arabinose-inducible expression vector pBAD33. A) The induction level of each RR after addition of the indicated concentration of arabinose was measured by immuno-blotting using anti-FLAG tag antibody. High-level induction of all six RRs was observed in the presence of 0.002% arabinose. B) In this condition, phosphorylation state of NarL, NarP, RcsB and UvrY was observed by Zn²⁺Phos-tag SDS-PAGE and immuno-blotting using anti-FLAG tag antibody. White and Black arrow indicate non-phosphorylated and phosphorylated state respectively.

noncognate promoters examined: characteristic induction condition for EvgA and UhpA; over-expression for NarL, NarP, RcsB and UvrY. However, NarL, NarP, RcsB and UvrY overexpression under these condition is not clear whether be phosphorylated or not. Zn²⁺Phos-tag SDS-PAGE permits the separation of phosphoproteins from their nonphosphorylated counterparts, therefore radiolabel-free profiling of protein phosphorylation is possible (Kinoshita *et al.* 2014). This methodology is frequently used for recent study of TCS phosphorylation. Using Zn²⁺Phos-tag SDS PAGE and western blotting by anti-FLAG antibody, rate of phosphorylated forms of the FLAG-tagged NarL, NarP, RcsB and UvrY to their all forms *in vivo* were determined; NarL is 49.6%, while NarP is 0%, RcsB is 4% and UvrY is 2.3% (Fig. 1-B). Unphosphorylated RRs are, however, generally able to bind to target DNA and activate transcription if they are present at high concentrations. For example, overexpressed wild-type UhpA or UhpA mutant with the mutation on the conserved Asp residue activate cognate *uhpT* promoter with or without G6P signal (Webber & Kadner, 1997).

2-3-3. Reporter assay of NarL-family RR-dependent promoters

As an attempt to detect possible cross talk *in vivo* at stage 3 of the signal transduction of *E. coli* TCSs, an effort was made in this study to test the cross-recognition between NarL-family RRs and their target promoters. For detection of the promoter activity, we applied two reporter assay systems using LacZ (β -galactosidase) and Lux (luciferase). For construction of the single-copy LacZ reporter assay system, all the test promoters were fused to the *lacZ* coding frame within the transfer plasmid, and the promoter-*lacZ* fusion was then inserted into the *E. coli* genome via lambda phage (Simons *et al.* 1987). As the activity of *uhpT-lacZ* translational fusion protein was low, we also used the Lux reporter system. RR-dependent promoter-lux transcription fusions were inserted into the pLUX vector using In-Fusion HD cloning kit (Clontech) (see Table 3). Using these two reporter systems, the activity of test promoters was examined under two different expression conditions of RRs.

2-3-4. Cross talk *in vivo* in promoter recognition between six NarL-family RRs

The stage 3 cross talk *in vivo* was examined for a total of 30 combinations between six RRs and five representative promoters [note that NarL and NarP recognize the same *nirB* promoter] under the induction condition characteristic to each RR or in the presence and absence of over-expressed RRs. The expression of reporter LacZ was observed for the authentic combination of EvgA-*ydeP* promoter (Fig. 2A, lane 1), NarL-*nirB* promoter (Fig. 2B, lane2), NarP-*nirB* promoter (Fig. 2C, lane 2), RcsB-*wza* promoter (Fig. 2D, lane 3), and UvrY-*csrB* promoter (Fig. 2F, lane 5). The expression of *uhpT-lacZ* translational fusion was, however, not observed in the presence of all six RRs including UhpA. With the use of *uhpT-lux* transcription fusion, UhpA-dependent expression of the *uhpT* promoter was detected at a significant level (Fig. 2E, lane 4).

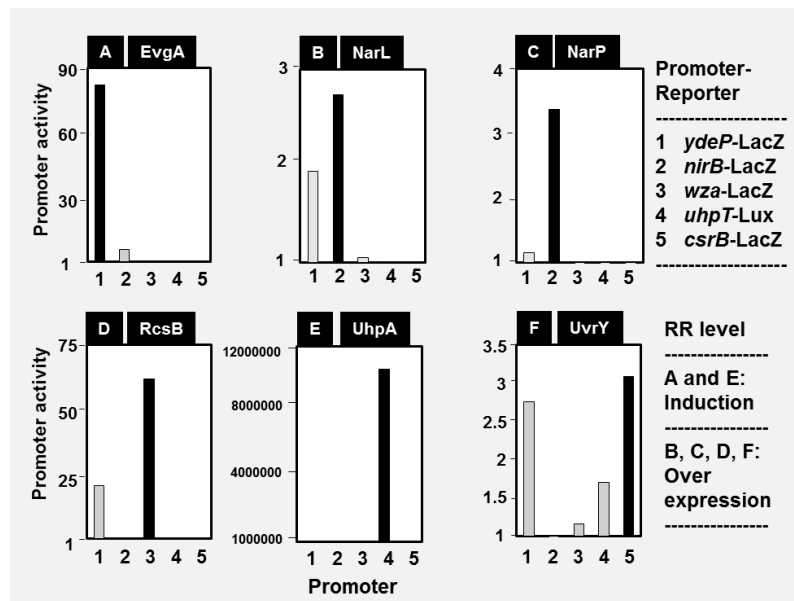


Fig. 2. Detection of RR-dependent expression of the regulation target promoter. For all six NarL-family RRs, one representative regulation target gene was selected. The promoter activity was measured using the reporter assay system of either LacZ or Lux reporter as indicted on right side. The increased level of RRs was achieved by either external signal-dependent induction or by artificial over-expression using the arabinose-inducible expression vector as indicated on right side. Promoter activity shows that the activity ratio with signal/ without signal or RR expression plasmid/vector). The reporter activity was detected for some non-cognate combinations of test RR and the promoter. Black and grey bar shows promoter activation by known and unkown combination, respectively.

Table 5. Cross-talk Identified by Reporter Assay

	EvgA	NarL	NarP	RcsB	UhpA	UvrY
<i>ydeP</i>	+	+	-	+	-	+
<i>nirB</i>	-	+	+	-	-	-
<i>wza</i>	-	-	-	+	-	-
<i>uhpT</i>	-	-	-	-	+	+/-
<i>csrB</i>	-	-	-	-	-	+

Transcription activation of five test promoters, shown on left-side column, by six NarL-family RRs, shown on top line, was examined by using the reporter assay with use of two reporters, LacZ (β -galactosidase) and Lux (luciferase). Gray shows cognate and hitherto identified combination of RR and promoter.

The highest level of reporter expression was always observed when the reporter was under the control of each cognate RR. The recognition of noncognate promoters was observed only for a limited combination between six NarL-family TFs and five promoters. NarL recognizes not only its cognate promoter *nirB* but also a noncognate promoter *ydeP* (Fig. 2B). Likewise, RcsB recognizes not only its cognate promoter *wza* but also a noncognate promoter *ydeP* (Fig. 2D). Between the six RRs, the wide spectrum of promoter recognition was observed with UvrY, recognizing *ydeP* and *uhpT* besides its cognate promoter *csrB* (Fig. 2F). Among the total of 30 combinations of RR-promoter interplay, the cross talk was significantly detected for four cases (Table 5), among which the *ydeP* promoter is unique, because it is recognized by not only its cognate RR EvgA but also three non-cognate RRs (NarL, RcsB, and UvrY) (Fig. 2). Therefore, the expression of YdeP might be induced in response to varieties of the environmental signal such as an acidic condition (through EvgSA), in the presence of nitrate/nitrite (through NarXQ-NarL and NarXQ-NarP) or in the presence of carboxylic acid, *etc.* formate and acetate (through BarA-UvrY). EvgSA constitutes a complex activation cascade including a number of downstream TFs (Eguchi & Utsumi 2014), but the constitutive resistance to acid pH is established after expression of only the acid resistance protein YdeP with oxidoreductase domain (Johnson *et al.* 2014).

The transporter protein UhpT is needed to acquire phosphorylated sugars from the environment for utilization as carbon and/or energy sources, and its expression depends on the UhpABC system (Kadner *et al.* 1994). The *uhpT* promoter was activated by not only its cognate UhpA but also a noncognate UvrY (see Table 2). BarA and UvrY are known to form a TCS pair (Pernestig *et al.* 2001), which is induced by the metabolic end products formate and acetate (Chavez *et al.* 2010).

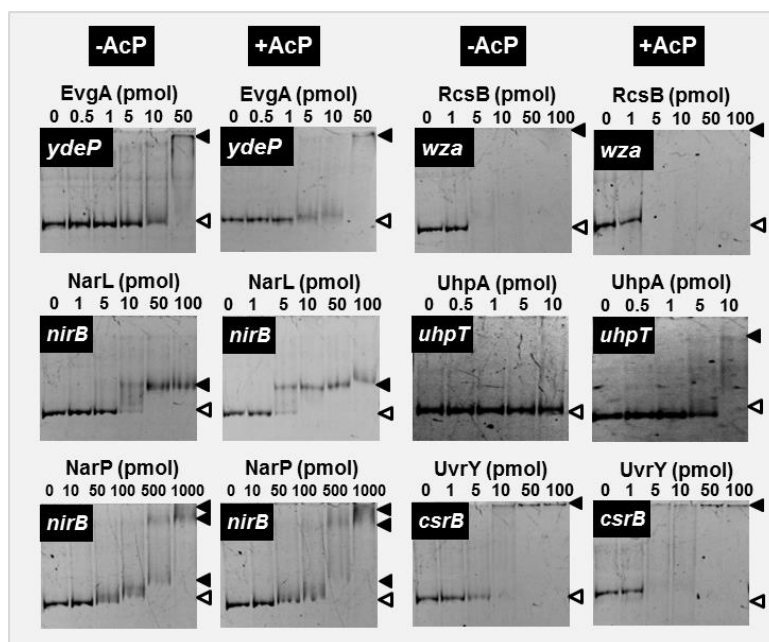


Fig. 3. Detection of the promoter-RR complex formation in vitro between cognate combinations. The promoter-binding activity of all six NarL-family RRs to the respective cognate promoters was examined using the gel shift assay. The RR-promoter complex formation was observed for all six combinations. In the presence of acetyl phosphate for in vitro phosphorylation of RRs, the promoter-binding affinity was significantly enhanced for EvgA, NarL, UhpA, and UvrY.

2-3-5. Cross talk *in vitro* in promoter recognition between six NarL-family RRs

The promoters that exhibited the cross talk should carry the recognition sequence recognized by each of the noncognate RRs. To confirm the observed cross talk between NarL-family RRs and the test promoters, we next tested the binding *in vitro* of six RRs to each of five representative promoters. For this purpose, all six RRs were over-expressed as His-tagged forms using the respective expression plasmids and affinity-purified apparently to homogeneity according to the standard procedure (Yamamoto *et al.* 2005). The binding of purified RRs to DNA probes, each including the cognate promoter, in the presence and absence of acetyl phosphate (AcP) for RR activation was examined by the gel shift assay. All RRs formed slowly migrating probe-protein complexes upon increase of protein concentration in the presence and absence of AcP (Fig. 3). In the presence of AcP, however, the binding affinity to the cognate promoters significantly increased for EvgA, NarL, UhpA and UvrY, but little enhancement was observed for NarP and RcsB. The observed difference in the minimum amount of RR needed for effective DNA binding might be due to the difference in affinity to the promoter or the difference in the level of functional RR form. In the case of RcsB, the

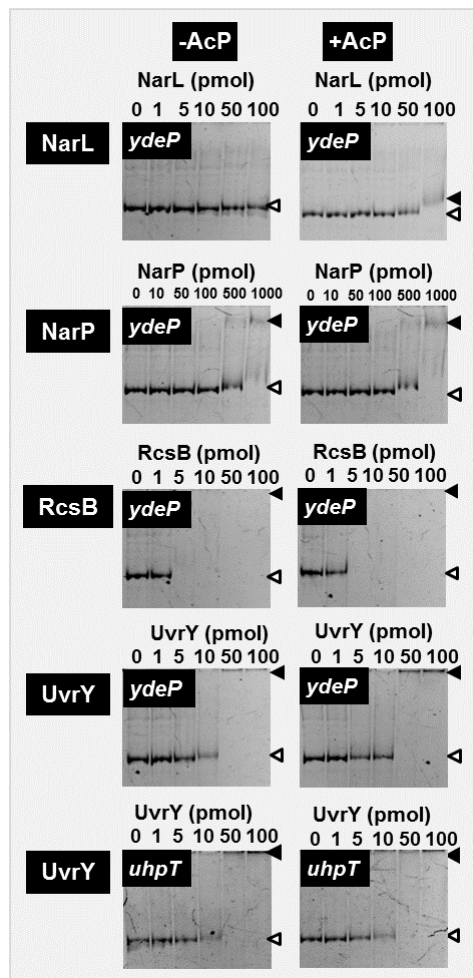


Fig. 4. Detection of the promoter-RR complex formation *in vitro* between non-cognate combinations. The promoter-binding activity of all six NarL-family RRs to the non-cognate promoters was examined using the gel shift assay. Among the total of 24 combinations (6 RRs versus 4 promoters), the formation of RR-promoter complex was identified for 5 cases: NarL-*ydeP* promoter; NarP-*ydeP* promoter; RcsB-*ydeP* promoter; UvrY-*ydeP* promoter; and UvrY-*uhpT* promoter.

activity level *in vivo* is controlled under the multicomponent RcsF/RcsC/RcsD/RcsB-RcsB phosphorelay (Majdalani & Gottesman 2005; Castanie-Cornet *et al.* 2007), and thus, the phosphorylation by AcP alone might not be enough for effective activation of its binding to the target promoter. Indeed, phosphorylation of RcsB by AcP *in vivo* requires RcsD in *Salmonella enterica* (Pescaretti *et al.* 2013).

Using these sets of RRs and promoters, I then tested cross talk in promoter binding for all 30 combinations between six RRs and five promoters. Except for the binding to the cognate target promoters, clear cross talk was observed for five combinations: NarL-*ydeP* promoter; NarP-*ydeP* promoter; RcsB-*ydeP* promoter; UvrY-*ydeP* promoter; and UvrY-*uhpT* promoter. The concentrations of RRs needed for effective binding to noncognate promoters were generally higher than those to the cognate promoters (compare Figs 3, 4), indicating that the binding affinity to noncognate promoters is lower than that to the cognate promoters. NarL and NarP recognize same sequence, although NarP had no effect on the *ydeP* activity *in vivo*, while NarL activated *ydeP*. NarP may needs a condition differ from this study for the *ydeP* activation.

The binding of NarL to *ydeP* promoter was significantly enhanced in the presence of AcP, but the enhancing role of AcP was not so significant for other four combinations. *In vivo* assay, also significant phosphorylation of RcsB and UvrY was not observed, while phosphorylation of NarL was observed. Therefore, NarP, RcsB and UvrY might activate *ydeP* independent to their phosphorylation. The combinations of cross talk in RR-promoter complex formation *in vitro* are in good agreement with the cross talk detected *in vivo* using the reporter assay (see Table 5).

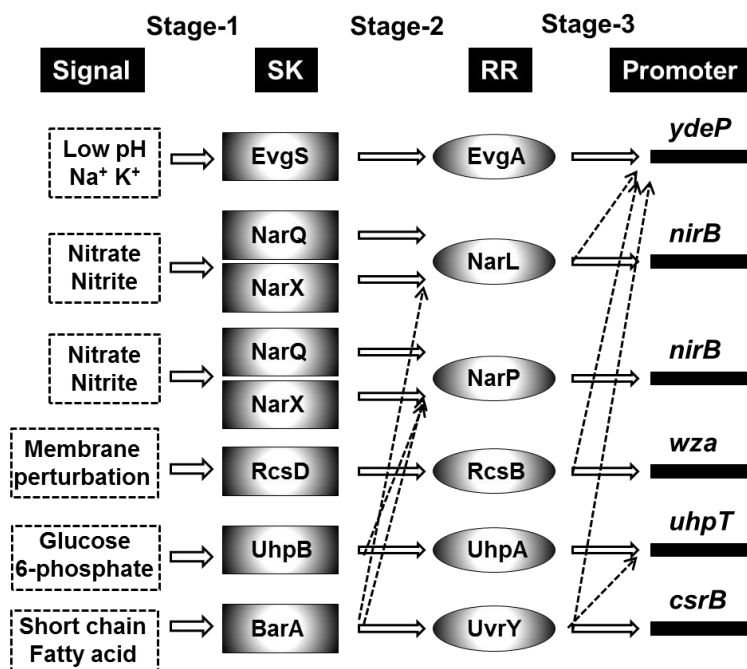


Fig 5. Cross-talks between NarL-family in the three stages of signal transduction. Cross talks at stage-1 was predicted using the hitherto published induction factors to each TCS while the cross-talks at stage-2 are described in Tyson *et al.* (1994) and Yamamoto *et al.* (2005). In this study, 4 cases of the stage-3 cross-talk for six NarL-family RRs is identified.

2-4. Discussion

2-4-1. Multifactor promoters

Among the NarL-family RR-dependent promoters analyzed in this study, the *ydeP* promoter was found to be activated by four RRs, EvgA, NarL, RcsB, and UvrY (Fig. 5A). These findings suggested the presence of recognition sequences on this *ydeP* promoter by these four RRs. In fact, the recognition sequences of not only EvgA but also NarL/NarP, RcsB and UvrY were identified (Fig. 6A). In addition, the general silencer H-NS-binding site was identified in the *ydeP* promoter region (Shimada *et al.* 2011), and NagC- and PhoP-binding sequences are known to exist in the *ydeP* promoter region (RegulonDB).

Increased numbers of the promoter have been identified to be under the control of multiple TFs. The genes encoding the global regulators generally carry the ‘multifactor promoters’ such as *csgD* encoding the master regulator of biofilm formation (Ogasawara *et al.* 2010, 2011; Ishihama 2012), *sdiA* coding for the key regulator of cell division (Shimada *et al.* 2013), and *flhDC* encoding the master regulator of flagella formation (Clarke & Sperandio 2005; Ogasawara *et al.* 2011) which are under the control of multiple transcription factors. For instance, more than 20 regulators are involved in the regulation of the *csgD* promoter (Ishihama 2012). Along this line, the *ydeP* promoter could be classified into the group of multifactor promoters.

YdeP is putative formate dehydrogenase oxidoreductase alpha (molybdopterin) subunit. Formate dehydrogenase plays an important role for anaerobic respiration. NarX-NarL respond to high-level nitrate and nitrite and then regulates genes involved in anaerobic electron transport and fermentation. YdeP might be needed for anaerobic respiration respond for high-level nitrate and nitrite. YdeP is also known to be necessary for *E. coli* acid resistance (Johnson *et al.* 2014). BarA-UvrY responds to short-chain fatty acid, such as formate and acetate, and regulates the various cell processes such as carbon storage, biofilm formation, virulence and motility. During the log phase, environment become acidic due to *E. coli* consume the sugar and dissipate acetate. Therefore, in response to acetate, UvrY up-regulate *ydeP* expression for acid resistance and acts for coassimilate both acetate and the remaining sugar before the sugar is exhausted. In addition to these, RcsC-RcsD-RcsB responds to membrane perturbation and regulates the biofilm formation and acid resistance. Not only by phosphorylation, but also RcsB act as heterodimer with various TF regardless for its phosphorylation (Pannen *et al.* 2016). RcsB is suggested that forms heterodimer with several NarL-family TFs containing EvgA, NarL, UvrY (Pannen *et al.* 2016). Through the regulation by RcsB, YdeP may be able to response to the multiple signal.

Moreover, TF YdeO expressed from downstream promoter of *ydeP*, also participate to regulation both of acid resistance and anaerobic respiration (Yamanaka *et al.* 2014). In this study, *ydeO* is revealed that also regulated by not only cognate RR EvgA but also NarL, RcsB and UvrY (data is not shown). Take all result together, cross-regulation of *ydeP* and *ydeO* promoter by EvgA, NarL, RcsB and UvrY plays the important role for acid resistance and anaerobic respiration.

2-4-2. Cross talk at TCS stage 3

Using NarL-family as model systems, I established that the stage 3 cross talk takes place for limited

combinations of RRs and their regulation target promoters. In the Zn^{2+} Phos-tag SDS-PAGE experiment, overexpressed RcsB and UvrY that activated *ydeP* promoter *in vivo* appeared to be not phosphorylated. Binding of RcsB and UvrY to *ydeP* promoter *in vitro* were also not affected by acetylphosphate. It is suggested that unphosphorylated RcsB and UvrY recognize *ydeP* promoter. Previous report shows that, in *Salmonella*, over-expression of RcsB D56Q mutant which is confirmed that is not able to be phosphorylated induces *csgD* expression (Latasa *et al.* 2012). Moreover, over-expression of UhpA mutant which possesses mutation on the conserved Asp residue had been also reported that induce cognate *uhpT* promoter expression (Webber & Kadner, 1997). Some of RRs might function as unphosphorylated form under the over-expressed condition. Recent study shows that necessity of phosphorylation and dimerization of RR for binding to promoter is different between the promoters with different affinity (Katsir *et al.* 2015).

Under *in vivo* situations, a set of RRs is activated directly by AcP but independent of HK–RR pathway (McCleary & Stock 1994; Pruss & Wolfe 1994). For instance, sugar-phosphate transporter UhpT is expressed in the absence of extracellular glucose-6-phosphate and in the absence of UhpBC (Verhamme *et al.* 2002a, b). This constitutive expression of UhpT is attributable to activation of UhpA by AcP. The intracellular concentration of AcP is strongly dependent on the metabolic state of the cell, as well as on growth phase, carbon source, pH, and temperature (McCleary & Stock 1994; Pruss & Wolfe 1994). Therefore, the concentration of cytoplasmic AcP can be regarded as a physiologically relevant signal, feeding into the signal transduction systems of *E. coli*. Transcription of *uhpT* was dependent on the growth phase of the cells, because the intracellular AcP concentration is growth phase dependent (McCleary & Stock 1994; Pruss & Wolfe 1994). The growth on pyruvate possibly results in high intracellular levels of AcP. This metabolite readily phosphorylates several RRs *in vitro* and *in vivo* (McCleary *et al.* 1993; McCleary & Stock 1994). The rates of phosphorylation and dephosphorylation of RRs by cognate HKs are different between RR species (Yamamoto *et al.* 2005), and thus, the metabolic stability of phosphorylated RRs might be different between RR species. Accordingly, the content of phosphorylated form in purified RR preparations is different between different RRs as detected using Phos-tag assay (Barbieri & Stock 2008).

On the basis of the observations herein described, I propose that the cross talk takes place at all three stages in the TCS signal transduction pathway. Exploiting the large number of sequenced bacterial genomes and an operon structure which packages many pairs of interacting TCS proteins together, a computational approach was developed to extract a molecular interaction, as many as 15–25% of the TCS proteins have been proposed to participate in out-of-operon cross talks (Procaccini *et al.* 2011). For the stage 2 in the TCS signal transduction, however, mathematical models shows that introducing cross talk always decreases system performance (Rowland & Deeds, 2014). It have been also considered that to avoid deleterious cross talk, selective pressure influence the evolution of the protein interaction interfaces immediately post duplication (Capra *et al.* 2012). Most of stage 2 cross talk that had been reported hitherto, indeed, observed only under the cognate SK deleted situation. In these cases, it is appears that cross talk *in vivo* is prevented by phosphatase activity of cognate or other SK, or preferential phosphate transfer to the cognate RR. For the cross talk at the stage 3, perhaps, recognition of the same sequence ambiguously between different RRs is also harmful to the cells. In this study, however, stage 3 cross talk are observed in limited combinations and

those seems to be specific. If the cross talk is specific, it may be especially important as a way of directly linking different regulation systems in a network to coordinate cell growth and metabolism.

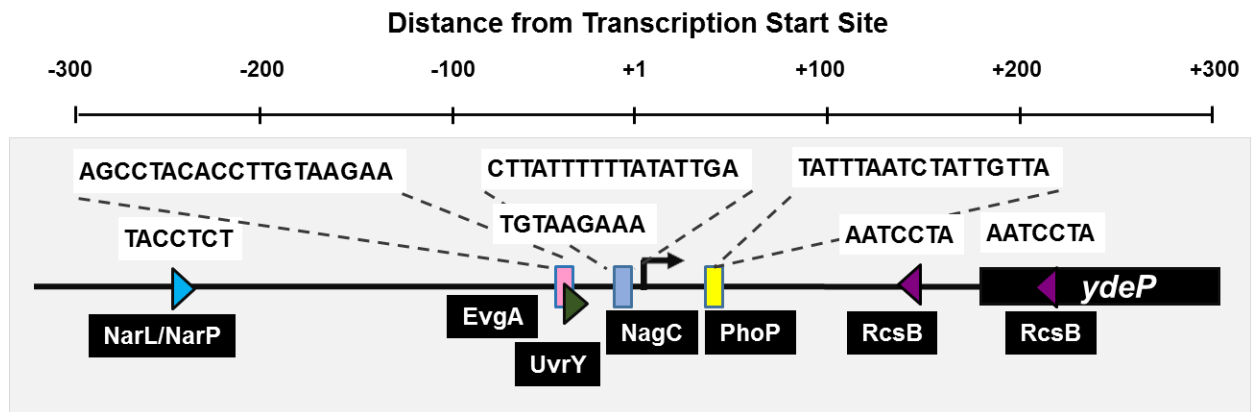


Fig 6. Location of TF-binding sites on the *ydeP* promoter.

CHAPTER3

CROSS TALK IN PROMOTER RECOGNITION BETWEEN FOURTEEN-OMPR FAMILY RESPONSE REGULATORS OF *ESCHERICHIA COLI* TWO-COMPONENT SYSTEM

3-1. Introduction

I proposed that the cross talk takes place at stage 3 in the TCS signal transduction pathway using six NarL-family RRs as a model system. In the same way, to determine how much frequency stage 3 cross talk occurs between OmpR-family RRs, I also analyze the cross-regulation between fourteen members of the OmpR-family RRs: ArcA (aerobic respiratory control), BaeR (bacterial adaptive response), BasR (bacterial adaptive response) also called PmrA (polymyxin-resistant), CpxR (conjugative plasmid gene expression), CreB (carbon source response), CusR (Cu-sensing regulator), KdpE (deleted in the K-dependent mutant), OmpR (outer membrane protein regulator), PhoB (phosphorus uptake and metabolism regulator), PhoP (response to low extracellular levels of divalent cation), QseB (quorum-sensing *E. coli* regulator B), RstA (regulator involved in different biological processes), TorR (TMAO reductase structural gene), YedW (Response for H₂O₂). For each of these fourteen OmpR-family RRs, the cross recognition *in vivo* of noncognate promoters were examined.

3-2. Materials and Methods

3-2-1. *E. coli* strains and growth conditions

Escherichia coli strains used in this study are summarized in Table 1. *E. coli* W3110 type-A was used for preparation of the expression clones of RRs, and of the regulation target promoters. *E. coli* BW25113 was used for the reporter assays of promoters under the control of test TFs. Cells were cultured in LB medium at 37°C. When necessary, 100 µg/mL ampicillin, 25 µg/mL kanamycin, or 20 µg/mL chloramphenicol was added into the medium. Cell growth was monitored by measuring the optical density at 600 nm.

3-2-2. Construction of arabinose-inducible expression system of RRs

The coding sequences of OmpR-family RR (ArcA, BaeR, BasR, CpxR, CreB, CusR, KdpE, OmpR, PhoB, PhoB, QseB, RstA, TorR and YedW) were PCR amplified using 5'-proximal and 3'-proximal primers of each open reading frame (for the sequence, see Table 2). Into all the 5'-primer sequence, the typical ribosome recognition sequence (SD sequence) was added. In addition, all the 3'-primers included a FLAG-tag sequence so as to be expressed as fusion with RRs. The PCR-amplified FLAG-tagged RR-coding sequences were inserted into *Sac* I and *Hind*III treated pBAD33 to construct the arabinose-inducible expression plasmids of FLAG-tagged RRs by In-Fusion HD (Clontech). The plasmid construct was confirmed by DNA sequencing (Table 3).

Table 1. Bacterial strains, bacteriophage

Bacterial strains		
<i>Escherichia coli</i> W3110 type-A	F ⁺ IN(<i>rrnD-rrnE</i>) <i>rph-1</i>	Jishage & Ishihama (1996)
<i>Escherichia coli</i> DH5α	F ⁺ <i>endA1 supE44 thiE1 recA1 gyrA96 deoR481 phoA8</i>	
<i>φ80ΔlacZ(M15) ΔhsdR17 (rK- mK+) Δ(argF-lac)169</i>		
<i>Escherichia coli</i> MC4100	F ⁺ <i>araD139 Δ(argF-lac)169 flhD5301 thiA1 relA1 rpsL150</i>	
<i>ptsF25 rbsR22 deoC1Δ(fimB-fimE)</i>	Casadaban (1976)	
<i>Escherichia coli</i> BW25113	F ⁺ <i>rrnB3ΔlacZ4787 hsdR514Δ(arabAD)567Δ(rhaBAD)568 rph-1</i>	Datsenko <i>et al.</i> (2000)
<i>Escherichia coli</i> TAICD	BW25113 <i>λicd-lacZ</i>	This study
<i>Escherichia coli</i> KBW1035	BW25113 <i>λspy-lacZ</i>	Yamamoto <i>et al.</i> (2008)
<i>Escherichia coli</i> TON1645	BW25113 <i>λais-lacZ</i>	Provided by H. Ogasawara
<i>Escherichia coli</i> TON1640	BW25113 <i>λyibD-lacZ</i>	Provided by H. Ogasawara
<i>Escherichia coli</i> TACPXP	BW25113 <i>λcpxP-lacZ</i>	This study
<i>Escherichia coli</i> BW25113/ <i>cusC-lacZ</i>	BW25113 <i>λcusC-lacZ</i>	Provided by T. Oshima
<i>Escherichia coli</i> TAKDPA	BW25113 <i>λkdpA-lacZ</i>	This study
<i>Escherichia coli</i> TAOMPC	BW25113 <i>λompC-lacZ</i>	This study
<i>Escherichia coli</i> TAPHOA	BW25113 <i>λphoA-lacZ</i>	This study
<i>Escherichia coli</i> KM4001	BW25113 <i>λmgtA-lacZ</i>	This study
<i>Escherichia coli</i> YY0304	BW25113 <i>λygiW-lacZ</i>	This study
<i>Escherichia coli</i> BW <i>φasr-lacZ</i>	BW25113 <i>λasr-lacZ</i>	Ogasawara <i>et al.</i> 2007
<i>Escherichia coli</i> YK0913	BW25113 <i>λtorC-lacZ</i>	This study
<i>Escherichia coli</i> BW25113/ <i>yedX-lacZ</i>	BW25113 <i>λyedX-lacZ</i>	Provided by T. Oshima
Bacteriophages		
λRS45	<i>bla-lacZ imm21 ind</i>	Simons <i>et al.</i> (1987)

Table 2. Oligonucleotide

Used for reporter plasmid		
ICD-P-LF	GGGATGAATTCTTTTAATGTTTTCGCTCCG	This study
ICD-P-LR	TCTCGGGATCCGCGTCGACCACTTTCAGCA	This study
CPXP-P-LF	CTCCAGAATTCACGGCGCAGGATCGCGCGA	This study
CPXP-P-LR	GATGTGGATCCAAACTTATGCCGTCGAACA	This study
KDPA-P-LF	GCGCGGAATCCAGAAAAGCATGAAAGGC	This study
KDPA-P-LR	GGTCAGGATCCCCAAGTGCGCGAAAAAGTA	This study
OMPC-P-LF	ATGTTGAATTCCTGTGAAATAGTTAACAAG	This study
OMPC-P-LR	TCTGGGGATCCTCTACATCTTTGTTGTCAG	This study
PHOA-P-LF	TATTTGAATTCGAGAAACGTTTCGCTGGTA	This study
PHOA-P-LR	GAGCGGGATCCTGATCACCCGTTAAACGGC	This study
YGIW-P-LF	AGTAAGAATTCCTGTAAACGCGCTCTGTACA	This study
YGIW-P-LR	CGCGCGGATCCACCCAGGTGTCGTCACGCAGGG	This study
TORC-P-LF	GCATAGGATCCCCAACTGACACAAAATTCGG	This study
TORC-P-LR	GTTGAGAATTCGCGCGAGGTCGATTCGCC	This study
UHPT_LR_F1	TCGTCTTCACCTCGACTTTTGAACGCCAGACACCGCGC	This study
UHPT_LR_R1	ACTAACTAGAGGATCAGCCAGCATGGGTTACTCCTGAAAT	This study
Used for RR expression plasmid		
ARCAF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCATGCAGACCCC GCACATTCTTAT	This study
ARCAR-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGT AGTCATCTTCCAGATCACCGCAGA	This study
BAERF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCATGAAGTTCTG GCGACCCGGTAT	This study

Table 2. Continued

BAERR-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGT AGTCTACTTCTCTCTGTAAATCCC	This study
BASRF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCATGAAAATTCT GATTGTTGAAGA	This study
BASRR-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGT AGTCGTTTTCTCATTTCGCGACCA	This study
CPXRF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCATGAATAAAAT CCTGTTAGTTGA	This study
CPXRR-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGT AGTCTGAAGCAGAAACCATCAGA	This study
CREBF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCATGCAACGGGA AACGGTCTG	This study
CREBR-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGT AGTCCAGGCCCTCAGGCTATATC	This study
CUSRF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCATGAAACTGTT GATTGTCGAAGA	This study
CUSRR-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGTA GTCCTGACCATCCGGCACCTCAA	This study
KDPEF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCATGACAAACGT TCTGATTGT	This study
KDPER-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGT AGTCAAGCATAAACCGATAGCC	This study
OMPRF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCATGCAAGAGAA CTACAAGATTCT	This study
OMPRR-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGTA GTCTGCTTTAGAGCCGTCCGGTA	This study
PHOBF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCATGGCGAGACG TATTCTGGT	This study
PHOBR-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGT AGTCAAAGCGGCTTGAAAAACGAT	This study
PHOPF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCATGCGCGTACT GGTTGTTGA	This study
PHOPR-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGT AGTCGCGCAATTTCGAACAGATAGC	This study
QSEBF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCATGCGAATTTT ACTGATAGA	This study
QSEBR-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGT AGTCTTCTCACCTAATGTGTAAC	This study
RSTAF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCGTGAATGTTAT GAACACTAT	This study
RSTAR-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGT AGTCATTCCCATGCATGAGGCGCAA	This study
TORRF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCATGCCACATCA CATTGTTAT	This study
TORRR-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGT AGTCGCACACATCAGCGCTAAGA	This study
YEDWF-1	TAGCGAATTCGAGCTAGGAGGAATTCACCATGAAGATTCT ACTTATTGA	This study
YEDWR-1	CAAAACAGCCAAGCTTTACTATTTATCGTCGTCATCTTTGT AGTCTTTTTTTACCGCTACGAATG	This study

Table 3. Plasmids

pRS552	promoter-less <i>lacZ</i> , Ap ^r	Simons <i>et al.</i> (1987)
pBAD33	pACYC184 derived, P _{BAD} Cm ^r	Guzman <i>et al.</i> (1995)
pICD	pRS552 (<i>icd-lacZ</i>)	This study
pCPXP	pRS552 (<i>cpxP-lacZ</i>)	This study
pKDPA	pRS552 (<i>kdpA-lacZ</i>)	This study
pOMPC	pRS552 (<i>ompC-lacZ</i>)	This study
pPHOA	pRS552 (<i>phoA-lacZ</i>)	This study
pYGIW	pRS552 (<i>ygiW-lacZ</i>)	This study
pTORC	pRS552 (<i>torC-lacZ</i>)	This study
puhpT-luxL1	pLUX (<i>uhpT-lux</i>)	This study
pUB6070	<i>creB-lacZ</i>	Cariss <i>et al.</i> 2008
pBADArcA-FLAG	pBAD33, FLAG-tagged ArcA at C-terminus	This study
pBADBaeR-FLAG	pBAD33, FLAG-tagged NarL at C-terminus	This study
pBADBasR-FLAG	pBAD33, FLAG-tagged NarP at C-terminus	This study
pBADCpxR-FLAG	pBAD33, FLAG-tagged RcsB at C-terminus	This study
pBADCreB-FLAG	pBAD33, FLAG-tagged UhpA at C-terminus	This study
pBADCusR-FLAG	pBAD33, FLAG-tagged UvrY at C-terminus	This study
pBADKdpE-FLAG	pBAD33, FLAG-tagged EvgA at C-terminus	This study
pBADOmpR-FLAG	pBAD33, FLAG-tagged NarL at C-terminus	This study
pBADPhoB-FLAG	pBAD33, FLAG-tagged NarP at C-terminus	This study
pBADPhoP-FLAG	pBAD33, FLAG-tagged RcsB at C-terminus	This study
pBADQseB-FLAG	pBAD33, FLAG-tagged UhpA at C-terminus	This study
pBADRstA-FLAG	pBAD33, FLAG-tagged UvrY at C-terminus	This study
pBADTorR-FLAG	pBAD33, FLAG-tagged UhpA at C-terminus	This study
pBADYedW-FLAG	pBAD33, FLAG-tagged UvrY at C-terminus	This study

3-2-3. Western blot analysis

Expression and purification of His-tagged RRs were determined by Western blot system (Jishage & Ishihama 1995) using anti-RR antibodies that were raised in rabbits using purified RRs, whereas the expression level of FLAG-tagged RRs was determined by Western blot analysis using anti-FLAG antibody. After SDS-PAGE, proteins were transferred onto PVDF membrane using iBlot gel transfer system (Invitrogen). Membranes were washed with skim milk in TBS buffer and then treated with anti-RR or anti-FLAG antibodies. The antibodies bound were detected using HRP (horseradish peroxidase)-linked anti-mouse IgG antibody as the secondary antibody. The chemiluminescence was measured using LAS-4000 CCD camera (Fujifilm).

3-2-4. Reporter assay: LacZ system

For detection of the regulatory roles of six test RRs, a set of reporter assay strains were constructed. In brief, approximately 650-bp-long sequence between -500 and +150 with respect to the initiation codon of each regulation target gene was PCR-amplified using a pair of the primers (for the primer sequences, see Table 2). After digestion with *EcoRI* and *BamHI*, the promoter fragment was inserted into *EcoRI*- and *BamHI*-treated pRS552 (Simons *et al.* 1987), leading to construct a set of promoter assay vector (Table 3). For construction of the single-copy LacZ vector, these plasmids were transformed into *E. coli* MC4100, into which λ RS45 was infected. Resultant lysogenic phages were transfected into *E. coli* BW25113, and their lysogens, each carrying a single copy of test promoter-*lacZ* fusion, were used for the LacZ reporter assay.

The assay of β -galactosidase activity was carried out using the standard procedure (Simons *et al.* 1987). In brief, an aliquot of cell culture was mixed with a reaction mixture (Z-buffer plus SDS and chloroform) and then treated with ONPG (Yamamoto *et al.* 2011). The yellow color developed from ONPG was measured at 420 nm. The activity was expressed as the Miller unit.

3-3. Results

3-3-1. Test systems of the stage 3 cross talk of *E. coli* OmpR-family RRs

Specific stage 3 cross talk was observed between six NarL-family RRs. As an extension of this line research, I analyzed in this study the stage 3 cross talk between fourteen OmpR-family RRs: ArcB, BaeR, BasR, CpxR, CreB, CusR, KdpE, OmpR, PhoB, PhoP, QseB, RstA, TorR and YedW (Table 4-A).

ArcA functions for the response to changing respiratory conditions of growth. Under anaerobiosis, ArcA regulates a great number of operons involved in respiratory and fermentative metabolism, while aerobiosis oxidised forms of quinone electron inhibits ArcA activity (Gunsalus & Park 1994; Malpica *et al.* 2006; Alvarez & Georgellis, 2010). BaeR responds to alterations of the bacterial envelope (Bury-Mone *et al.* 2009) and regulates the genes involved in drug resistance and protein folding (Baranova & Nikaido, 2002; Nagakubo *et al.* 2002; Raffa & Raivio, 2002; Hirakawa *et al.* 2005; Nishino *et al.* 2005; Yamamoto *et al.* 2008). BasR response to elevated levels of Fe (III) which can permeabilize the outer membrane and result in cell death, leading to the transcriptional expression of several genes involved in modification of lipopolysaccharide to prevent excessive Fe (III) binding (Nagasawa *et al.* 1993; Chamnongpol *et al.* 2002; Yamamoto *et al.* 2005; Hagiwara *et al.* 2004). CpxR responds to multiple stimuli, such as alkaline pH, altered membrane lipid composition, interaction with hydrophobic surfaces, and high osmolarity, over-expression of outer membrane lipoprotein NlpE, adherence to hydrophobic surfaces and external copper ions (Dorel *et al.* 2006; Snyder *et al.* 1995; Otto & Silhavy, 2002; Gupta *et al.* 1995). CpxR regulates the expression of a great number of operons involved in the various cell functions, such as "conjugative plasmid gene expression" (cpx) (McEwen & Silverman, 1980; Lau-Wong *et al.* 2008), the envelope stress response system, pilus assembly, secretion, motility and chemotaxis, adherence, biofilm development, multidrug resistance and efflux (Dorel *et al.* 2006), and the copper-responsive regulatory system (Yamamoto & Ishihama, 2006). CpxR sometimes acts to modulate the action of the main activators or repressors of some promoters (Dorel *et al.* 2006). CreB responds to fermenting glycolytic carbon sources (Avison *et al.* 2001) and regulates the expression of genes involved in acetate, ribose and maltose metabolism, pentose phosphate pathway and repair DNA damage associated with the replication fork (Kakuda *et al.* 1994; Duun *et al.* 1999; Richet 1996; Sprenger, 1995; Saveson & Lovett, 1999). CusR is activated in anaerobic condition or extreme copper stress in aerobic condition (Outten *et al.* 2001) and regulates genes related to the copper and silver efflux systems (Munson *et al.* 2000; Franke *et al.* 2001). KdpE regulates the genes involved in a high-affinity potassium (K^+) uptake system P-type ATPase KdpFABC under K^+ -limiting conditions or under osmotic stress imposed by a salt (Voelkner *et al.* 1993; Altendorf *et al.* 1994; Jung *et al.* 2000; Jung & Altendorf, 2002; Ballal *et al.*

Table 4. OmpR-family RRs and their Promoters**A) Test response regulator**

SK	RR	Regulatory roles	Regulation target operons
ArcB	ArcA	Regulator for respiration and fermentation	<i>aceBAK, ackA-pta, appCBXA, cydAB, fnr, glpABC, icd</i> (77)
BaeS	BaeR	Regulator for drug resistans and protein folding	<i>acrD, mdtABCD-baeSR, spy, ycaC</i> (4)
BasS	BasR	Regulator for prevent excessive Fe (III) binding	<i>ais, csgDEFG, cspI, dgkA, fimB, putA, qseBC, tomB-hha, yibD</i> (12)
CpxA	CpxR	Regulator for the various cell functions	<i>acrD, cpxP, csgDEFG, degP, mdtABCD-baeSR, motAB-cheAW, ompC, ompF, rdoA-dsbA, rpoE-rseABC, rpoH, rprA, spy</i> (38)
CreC	CreB	Regulator for catabolic regulation	<i>creABCD, malEFG, nudF-yqiB-cpdA-yqiA-parE, talA-ktbB</i> (8)
CusS	CusR	Regulator for the copper and silver efflux systems	<i>cusCFBA, cusRS</i> (2)
KdpD	KdpE	Regulator for K ⁺ uptake system	<i>kdpFABC</i> (1)
EnvZ	OmpR	Regulator for outer membrane porin	<i>bdm-sra, bolA, csgDEFG, fadL, flhDC, ompC, ompF</i> (12)
PhoR	PhoB	Regulator for phosphorus uptake and metabolism	<i>adiC, amn, argP, asr, cra, cusCFBA, cusRS, phoA-psiF</i> (32)
PhoQ	PhoP	Regulator for Mg ²⁺ homeostasis	<i>acrAB, argD, borD, cysB, dcuD, envY-ompT, fadL, mgtLA</i> (36)
QseC	QseB	Regulator for flagella and motility	<i>flhDC, qseBC, ygiW</i> (3)
RstB	RstA	Regulator for different biological processes	<i>asr, csgDEFG, narGHJI, ompF</i> (4)
TorS	TorR	Regulator for TMAO induction	<i>gadAXW, hdeAB-yhiD, tnaCAB, torCAD, torR</i> (5)
YedV	YedW	Regulator for the copper and silver efflux systems	<i>cusCFBA, cusSR, cyoABCDE, huiH, yedVW</i> (5)

B) Test promoter

RR	Test promoter	Gene product	Protein function
ArcA	<i>icd</i>	Isocitrate dehydrogenase	Shift between TCA and glyoxalate pathways
BaeR	<i>spy</i>	ATP-independent periplasmic chaperone	Prevent protein aggregation and assists protein refolding
BasR	<i>ais</i>	LPS core heptose(II)-phosphate phosphatase	Modification of the lipopolysaccharide
BasR	<i>yibD</i>	Glucuronic acid transferase	Modification of the core oligosaccharide
CpxR	<i>cpxP</i>	Periplasmic adaptor protein	Inhibits the <i>cpx</i> response
CreB	<i>creD</i>	Inner membrane protein	Colicin-related functions
CusR	<i>cusC</i>	Copper/silver efflux system	Drug/analog sensitivity
KdpE	<i>kdpA</i>	Potassium translocating ATPase, subunit A	ATP-driven potassium ion transport
OmpR	<i>ompC</i>	Outer membrane porin protein C	Outer membrane constituents
PhoB	<i>phoA</i>	Bacterial alkaline phosphatase	Hydrolysis and transphosphorylation of phosphate monoesters
PhoP	<i>mgtA</i>	Magnesium transporter	Uptake of magnesium ion
QseB	<i>ygiW</i>	Periplasmic protein	Resistance for hydrogen peroxide and cadmium stress
RstA	<i>asr</i>	Acid shock-inducible periplasmic protein	Acid resistance
TorR	<i>torC</i>	Trimethylamine N-oxide (TMAO) reductase I	Cytochrome c-type subunit, anaerobic respiration
YedW	<i>yedX</i>	Hydroxyisourate hydrolase	Purine catabolism

2007). OmpR responds to changes in extracellular osmolarity and regulates the expression of several genes involved in major outer membrane porins OmpC and OmpF (Garrett *et al.* 1985; Nara *et al.* 1986; Mizuno & Mizushima, 1987; Csonka & Hanson, 1991; Maeda *et al.* 1991; Kanamaru & Mizuno, 1992; Huang & Igo, 1996; Cai & Inouye, 2002; Yoshida *et al.* 2006), flagella (Shin & Park, 1995), biofilm formation (Prigent-Combaret *et al.* 2001), curli (Vidal *et al.* 1998, Jubelin *et al.* 2005) and drug exporter genes (Hirakawa *et al.* 2003). PhoB is activated under the phosphate limitation conditions and then regulates genes involved in phosphorus uptake and metabolism (Wanner, 1993; VanBogelen *et al.* 1996; Baek & Lee, 2006). PhoP is activated in response to low extracellular levels of divalent cations such as magnesium or calcium (Groisman *et al.* 1992; Kasahara *et al.* 1992; Kato *et al.* 1999). PhoP activates expression of various genes involved in different biological processes, such as Mg²⁺ homeostasis, resistance to antimicrobial peptides, acid resistance,

and LPS modification (Kato *et al.* 1999; Minagawa *et al.* 2003; Miyashiro & Goulian, 2007). QseB responds to quorum-sensing, and then regulates transcription of genes involved in flagella and motility (Sperandio *et al.* 2002). RstA appears to be stimulated by low pH (Ogasawara *et al.* 2007; Yamamoto *et al.* 2005) and involved in different biological processes, such as acid tolerance, curli fimbria formation, and anaerobic respiration (Ogasawara *et al.* 2007). TorR is phosphorylated in the presence of TMAO (trimethylamine N-oxide) and then activates the genes related to TMAO induction (Pascal *et al.* 1991; Simon *et al.* 1994) and the enzymes of tryptophan metabolism and represses the genes of glutamate decarboxylase genes (Bordi *et al.* 2003). YedW responds to H₂O₂ and then regulates the same set of CusR target genes and promoters (Urano *et al.* 2015). It is considered that evolutionary origin of YedW and CusR is probably common (Bouzat & Hoostal, 2013).

As a model promoter for each of these fourteen RRs, I selected one representative gene from a set of the genes that have so far been identified as the regulation targets by each RR: *icd* for ArcA (Salmon *et al.* 2005; Park & Kiley, 2014); *spy* for BaeR (Raffa & Raivio, 2002; Yamamoto *et al.* 2008) [note that *spy* promoter is also regulated by CpxR through recognition different sequence (Yamamoto *et al.* 2006; Raivio *et al.* 2013)]; *ais* and *yibD* for BasR (Ogasawara *et al.* 2012; Froelich *et al.* 2006) [note that *yibD* promoter is also regulated by PhoB through recognition different sequence (Baek & Lee, 2006)]; *cpxP* for CpxR (Yamamoto *et al.* 2006; Raivio *et al.* 2013); *creD* for CreB (Avison *et al.* 2001; Cariss *et al.* 2008); *cusC* for CusR (Munson *et al.* 2000; Yamamoto & Ishihama, 2005) [note that *cusC* promoter is also recognized by PhoB and YedW with same sequence (Yang *et al.* 2012; Urano *et al.* 2015)]; *kdpA* for KdpE (Sugiura *et al.* 1992; Narayanan *et al.* 2012); *ompC* for OmpR (Mattison *et al.* 2002; Yoshida *et al.* 2006) [note that *ompC* promoter is also regulated by CpxR through recognition different sequence]; *phoA* for PhoB (Makino *et al.* 1986; Marzan *et al.* 2013); *mgtA* for PhoP (Minagawa *et al.* 2003); *ygiW* for QseB (Clarke & Sperandio, 2005) [note that *ygiW* promoter is also regulated by BasR through recognition different sequence (Guckes *et al.* 2013)]; *asr* for RstA (Ogasawara *et al.* 2007) [note that *asr* promoter is also regulated by PhoB through recognition different sequence (Suziedeliene *et al.* 1999)]; *torC* for TorR; and *yedX* for YedW (Urano *et al.* 2015) [note that *yedX* promoter is also regulated by CusR through recognition same sequence with YedW (Urano *et al.* 2015)] (Table 4–B).

3-3-2. Controll of the intracelluer level of fourteen OmpR-family RRs

For some of NarL-family RRs, high-level activation was not observed using the known induction conditions (refer to 2-3-1-2). In these cases, as yet unidentified factors or conditions might be needed for activation. On the other hand, overexpression of RR always induced cognate promoter expression regardless of their phosphorylation. Therefore, artificial over-expression of the test RRs were selected as the easily control system of the intracellular level of RRs. For the detection of the expression level, same to the NarL-family RRs, the RR genes were tagged with FLAG-tag sequence at 3'-terminus and then inserted into an arabinose-inducible expression vector. The arabinose concentration-dependent expression of FLAG-tagged RRs was confirmed by immunoblotting with use of anti-FLAG antibody (Fig. 1). The maximum level of expression was observed for all test RRs after the addition of 0.002% or 0.02% arabinose. Over-expression

of the test RRs were performed under the arabinose concentration that RR expression was induced with maximum level.

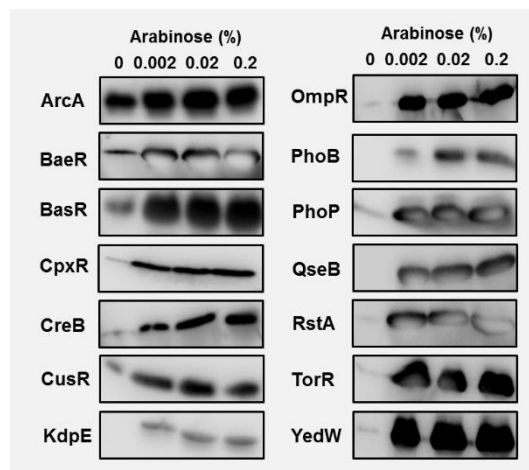


Fig. 1. Artificial expression of RRs. The coding sequences of fourteen OmpR-family RRs with FLAG-tag sequence at 3'-termini were inserted into the arabinose-inducible expression vector pBAD33. The induction level of each RR after addition of the indicated concentration of arabinose was measured by immuno-blotting using anti-FLAG tag antibody. High-level induction of RRs were observed in the presence of 0.002% or 0.02% arabinose.

3-3-3. Cross talk *in vivo* in promoter recognition between fourteen OmpR-family RRs

The stage 3 cross talk between OmpR-family RRs *in vivo* were examined for a total of 210 combinations between fourteen RRs and fifteen representative promoters in the presence and absence of over-expressed RRs. The expression of reporter LacZ was observed for the authentic combination of BaeR-*spy* promoter (Fig. 2B, lane 2), BasR-*ais* promoter (Fig. 2C, lane 3), BasR-*yibD* promoter (Fig. 2C, lane 4), CreB-*creD* promoter (Fig. 2E, lane 6), CusR-*cusC* promoter (Fig. 2F, lane 7), kdpE-*kdpA* promoter (Fig. 2G, lane 8), PhoB-*phoA* promoter (Fig. 2I, lane 10), PhoP-*mgfA* promoter (Fig. 2J, lane 11), RstA-*asr* promoter (Fig. 2L, lane 13) and YedW-*yedV* promoter (Fig. 2N, lane 15). The expression of *icd-lacZ*, *cpxP-lacZ*, *ompC-lacZ*, *ygiW-lacZ* and *torC-lacZ* were, however, not observed in the presence of all fourteen RRs (Fig. 2A, D, H, J, K, M). Although *icd* promoter is known to be repressed by ArcA, this repression could not be observed because *icd* activity was not detected at all. Because all of these reporter genes were constructed to translational fusion, LacZ might failed to be localized at cytoplasm due to locality of phused protein: CpxP is periplasmic protein; OmpC is outer membrane protein; YgiW is periplasmic protein and TorC is inner membrane protein. However, these promoter expression might be observed as transcriptional fusion likely to UhpA cognate promoter *uhpT*. UhpT is also inner memberane protein.

The recognition of noncognate promoters was observed only for a limited combination between fourteen OmpR-family TFs and 15 promoters, but the wide spectrum of promoter recognition was observed with many of OmpR-family RRs; ArcA, BaeR, CpxR, OmpR, QseB and YedW. ArcA activates noncognate

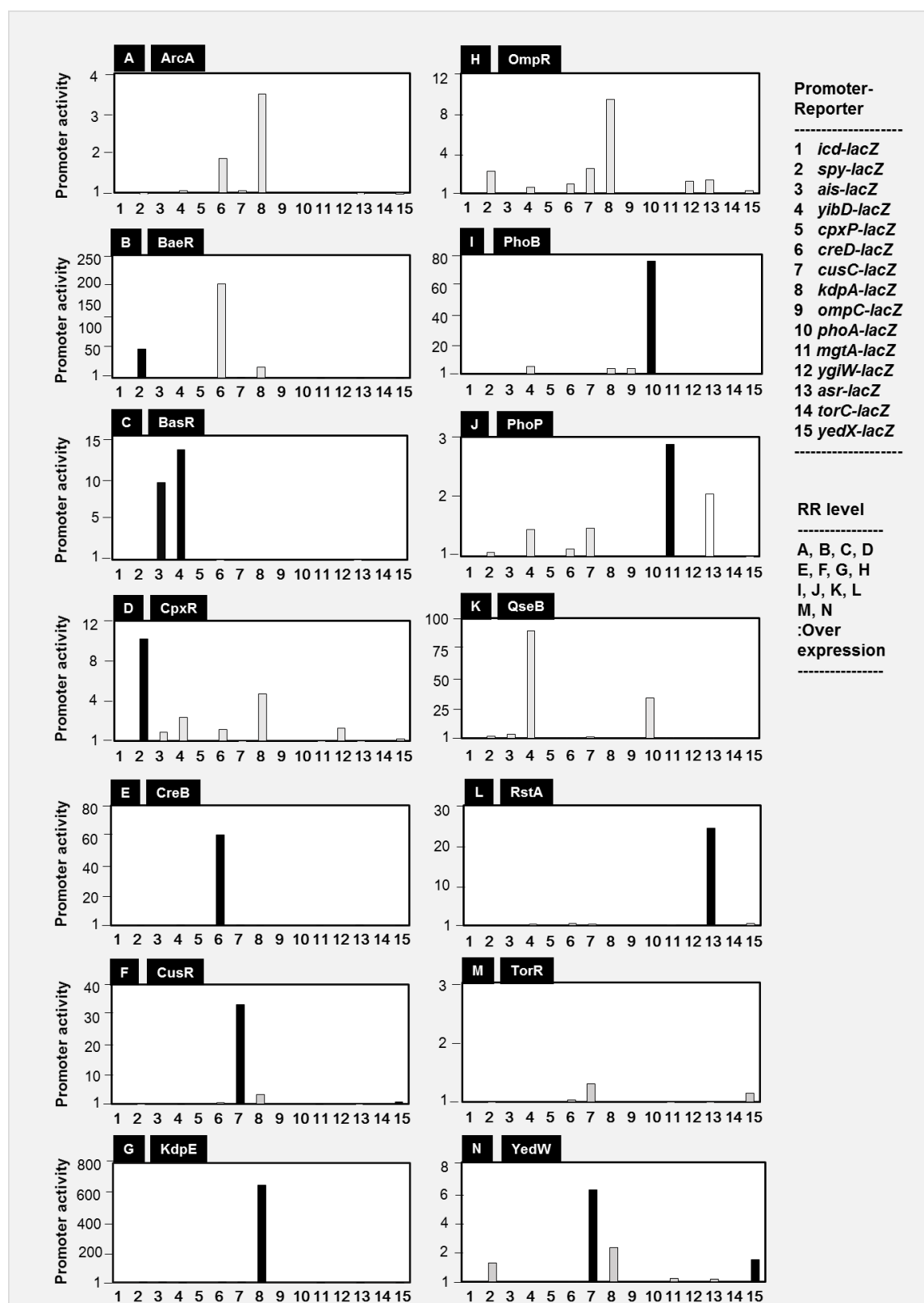


Fig. 2. Detection of RR-dependent expression of the regulation target promoter. For fourteen OmpR-family RRs, one representative regulation target gene was selected. The promoter activity was measured using the reporter assay system of either LacZ reporter as indicated on right side. The increased level of RRs was achieved by artificial over-expression using the arabinose-inducible expression vector as indicated on right side. Promoter activity shows that the activity ratio with signal/ without signal or RR expression plasmid/vector. The reporter activity was detected for some non-cognate combinations of test RR and the promoter. Black, grey and white bar shows promoter activation by combination of known, unknown and cascaded through cognate RR expression, respectively.

Table 5. Cross-talk Identified by Reporter Assay

	ArcA	BaeR	BasR	CpxR	CreB	CusR	KdpE	OmpR	PhoB	PhoP	QseB	RstA	TorR	YedW
<i>icd</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>spy</i>	-	+	-	+	-	-	-	+	-	-	-	-	-	+
<i>ais</i>	-	-	+	-	-	-	-	-	-	-	-	-	-	-
<i>yibD</i>	-	-	+	-	-	-	-	-	-	-	+	-	-	-
<i>cpxP</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>creD</i>	+	+	-	-	+	-	-	-	-	-	-	-	-	-
<i>cusC</i>	-	-	-	-	-	+	-	+	-	-	-	-	-	+
<i>kdpA</i>	+	+/-	-	+	-	-	+	+	-	-	-	-	-	+
<i>ompC</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>phoA</i>	-	-	-	-	-	-	-	-	+	-	+	-	-	-
<i>mgtA</i>	-	-	-	-	-	-	-	-	-	+	-	-	-	-
<i>ygiW</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>asr</i>	-	-	-	-	-	-	-	-	-	+	-	+	-	-
<i>torC</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>yedX</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Transcription activation of fifteen test promoters, shown on left-side column, by fourteen OmpR-family RRs, shown on top line, was examined by using the reporter assay with use of two reporters, LacZ (β -galactosidase). Gray shows cognate and hitherto identified combination of RR and promoter. Black shows cascade regulation via regulation of cognate RR gene expression.

promoter *creD* and *kdpA* while repression of cognate *icd* promoter was not observed (Fig. 2A). BaeR activates not only its cognate promoter *spy* but also a noncognate promoter *creD*, *kdpA* (Fig. 2B). CpxR activates noncognate promoter *spy* and *kdpA* while activation of cognate *cpxP* promoter was not observed (Fig. 2D). OmpR activates noncognate promoter *spy*, *cusC* and *kdpA* while activation of cognate *ompC* promoter was not observed (Fig. 2H). PhoP activates not only its cognate promoter *mgtA* but also a noncognate promoter *asr* (Fig. 2J). This activation, however, is known to be a cascade through induction of RstA expression. QseB activates noncognate promoter *yibB* and *phoA* while activation of cognate *ygiW* promoter was not observed (Fig. 2K). YedW activates not only its cognate promoter *yedX* but also *spy*, *cusC* and *kdpA* (Fig. 2N). However, although previous report shows that PhoB activates BasR cognate promoter *yibD* (Baek & Lee, 2006), CusR cognate promoter *cusC* (Yang *et al.* 2012), RstA cognate promoter *asr* (Suziedeliene *et al.* 1999) and YedW cognate promoter *yedX* (Yang *et al.* 2012), these cross talk by PhoB could not observed in this study. In these cases, phosphorylation of PhoB by phosphorus limitation is might needed.

Among the total of 210 combinations of RR-promoter interplay, the cross talk was significantly detected for 15 cases, 11 of them are the novel (Table 5). Among which the *creD*, *kdpA*, *spy* and *cusC* promoter are unique; *creD* recognized by not only its cognate RR CreB but also two non-cognate RRs, ArcA and BaeR; *kdpA* recognized by not only its cognate RR KdpE but also four non-cognate RRs, ArcA, CpxR, OmpR and YedW; *spy* recognized by not only its cognate RR BaeR but also three non-cognate RRs, CpxR, OmpR and

YedW; *cusC* recognized by not only its cognate RR CusR but also two non-cognate RRs, OmpR and YegW (Fig. 2).

Therefore, the expression of CreD might be induced in response to varieties of the environmental signal such as fermenting glycolytic carbon sources (through CreBC), under the anaerobic condition (through ArcAB) or envelop stress (through BaeSR). CreD over-expression induces tolerance to colicin E2 through CbrC overexpression (Drury & Buxton, 1988; Cariss *et al.* 2010). A high-affinity potassium (K^+) uptake system P-type ATPase KdpFABC is required for uptake of K^+ under K^+ -limiting conditions or under osmotic stress imposed by a salt, and its expression depends on the KdpDE system (Voelkner *et al.* 1993; Altendorf *et al.* 1994; Jung *et al.* 2000; Jung & Altendorf, 2002; Ballal *et al.* 2007). The *kdpFABC* promoter was activated by not only its cognate KdpE but also a noncognate BaeR, CpxR, OmpR and YedW (see Table 7). KdpFABC might be induced in response to envelop stress (through BaeSR and CpxAR), osmotic stress (through EnvZ-OmpR) and H_2O_2 stress (through YedVW). Spy prevent protein aggregation and assists protein refolding response to envelop stress (through BaeSR and CpxAR). Spy also may response to osmotic stress (through EnvZ-OmpR) and H_2O_2 stress (through YedVW). The expression of *cusC*, encodes copper/silver efflux system, suggested that responds to not only copper or silver ion (through CusSR), but also osmotic stress (through EnvZ-OmpR) and H_2O_2 stress (through YedVW).

Expression of glucuronic acid transferase YibD induced by alterations of the envelope (through BasSR) is might also induced by quorum-sensing (through QseCB). Expression of alkarine phosphatase PhoA induced by phosphorus limitation (through PhoRB) is might also induced by quorum-sensing (through QseCB).

3-4. Discussion

3-4-1. Multifactor promoters

Through the analysis of stage 3 cross talk between NarL-family, the *ydeP* promoter considered that be classified into the group of multifactor promoters. In this line, the *kdpA* and *spy* promoter also could be called to multifactor promoters.

Among the OmpR-family RR-dependent promoters analyzed in this study, the *kdpA* promoter was found to be activated by six RRs, ArcA, BaeR, CpxR, KdpE, OmpR and YedW, and the *spy* promoter was found to be activated by four RRs, BaeR, CpxR, OmpR and YedW (Fig. 3). On the *kdpA* promoter, the recognition sequences of not only KdpE but also ArcA, BaeR, CpxR, OmpR and YedW were identified (Fig. 4A). Also on the *spy* promoter, the recognition sequences of not only BaeR and CpxR but also OmpR and YedW were identified (Fig. 4B). For the *kdpA* promoter, identified recognition sequence of BaeR and one of three OmpR are contained in KdpE recognition sequence. In addition, some of ArcA and CpxR sequence are also overlapped. For the *spy* promoter, OmpR sequence is overlapped with BaeR binding site only partially. Recognition sequences of almost RRs are various but specific. According to slight differences of sequence, it is might be determine whether RRs recognize the sequence or not. However, it is necessary to confirm whether RRs bind to these sequences in fact.

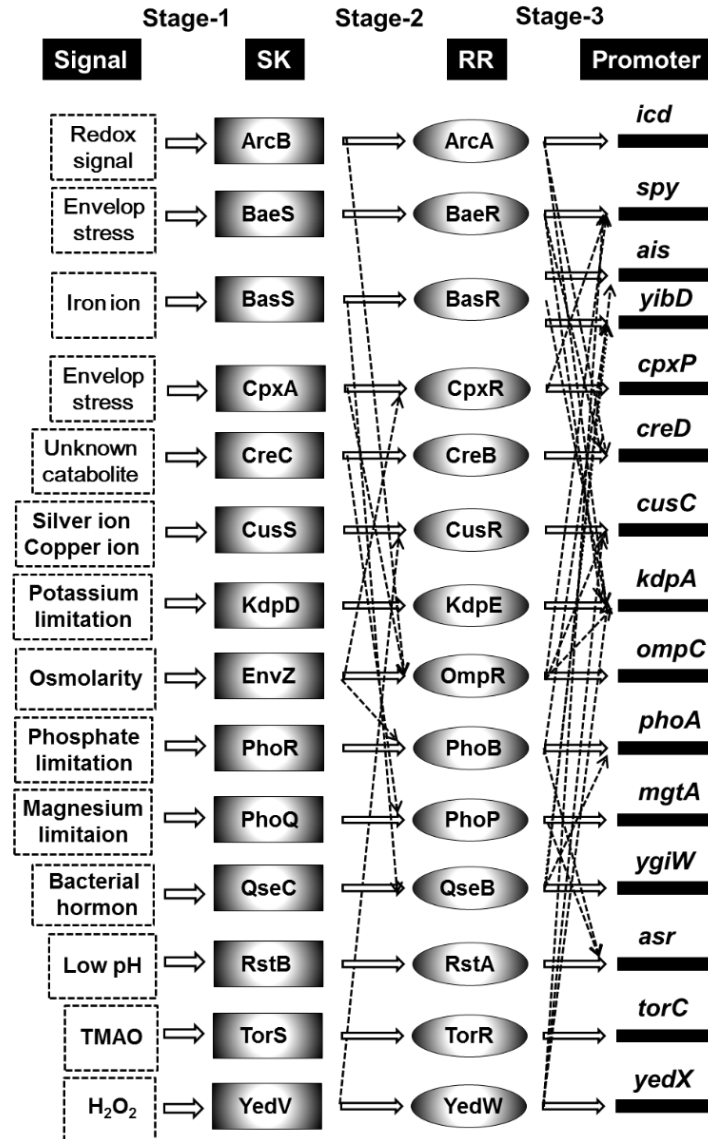


Fig 3. Cross-talks in the three stages of signal transduction between OmpR-family. Cross talks at stage-1 was predicted using the hitherto published induction factors to each TCS while the cross-talks at stage-2 are described in Wanner (1995), Kim *et al.* (1996), Matsubara *et al.* (2000), Yamamoto *et al.* (2005), Siryaporn & Goulian (2008) and Guckes *et al.* (2013). In this study, 15 cases of the stage-3 cross-talk for fourteen OmpR-family RRs is identified.

A high-affinity potassium (K⁺) uptake system P-type ATPase KdpFABC is required for uptake of K⁺ under K⁺-limiting conditions or under osmotic stress imposed by a salt. In other word, this system needs regulation respond to K⁺-limiting conditions or osmotic stress. And that may be realized through the *kdpA* promoter regulation by its cognate KdpDE (for K⁺-limiting conditions), noncognate EnvZ-OmpR (for osmotic stress), BaeSR, CpxAR and YedVW (for membrane perturbation including H₂O₂ stress which related to osmotic stress). Spy prevent protein aggregation and assists protein refolding response to envelop stress through BaeSR and CpxAR. It is also presumed that osmotic stress and H₂O₂ cause a membrane destruction. Therefore, Spy is also needed under such conditions and regulated by EnvZ-OmpR and YedVW.

3-4-2. Cross talk at TCS stage 3

Using OmpR-family RRs as model systems, I confirmed that the stage 3 cross talk takes place for limited combinations of RRs and their regulation target promoters. For OmpR-family RRs, stage 3 seem to be specific, although some of overlapped recognition between OmpR-family RRs (KdpE-OmpR-BaeR and ArcA-CpxR) are also expected differ from NarL-family RRs. That is, they shares a common motif. In the most case, however, ambiguous sequence recognition between RRs is not be presumed. In any case, stage 3 cross talk is suggested that specific and precise event which make TCS signal transduction complicated.

Phosphorylation is also involved in RR sequence recognition. Some of RRs might function as unphosphorylated form under the over-expressed condition. However, in this study, transcripion activation of *yibD*, *cusC*, *asr* and *yedX* by PhoB which had been reported previously could not be observed by artificial over-expression of PhoB, although overexpression of PhoB induced cognate promoter *phoA* expression. Recent study shows that necessity of phosphorylation and dimerization of RR for binding to promoter is different between the promoters with different affinity (Katsir *et al.* 2015). For the activation of *yibD*, *cusC*, *asr* and *yedX* promoter, phosphorylation of PhoB by phosphorus limitation stimulus might be needed.

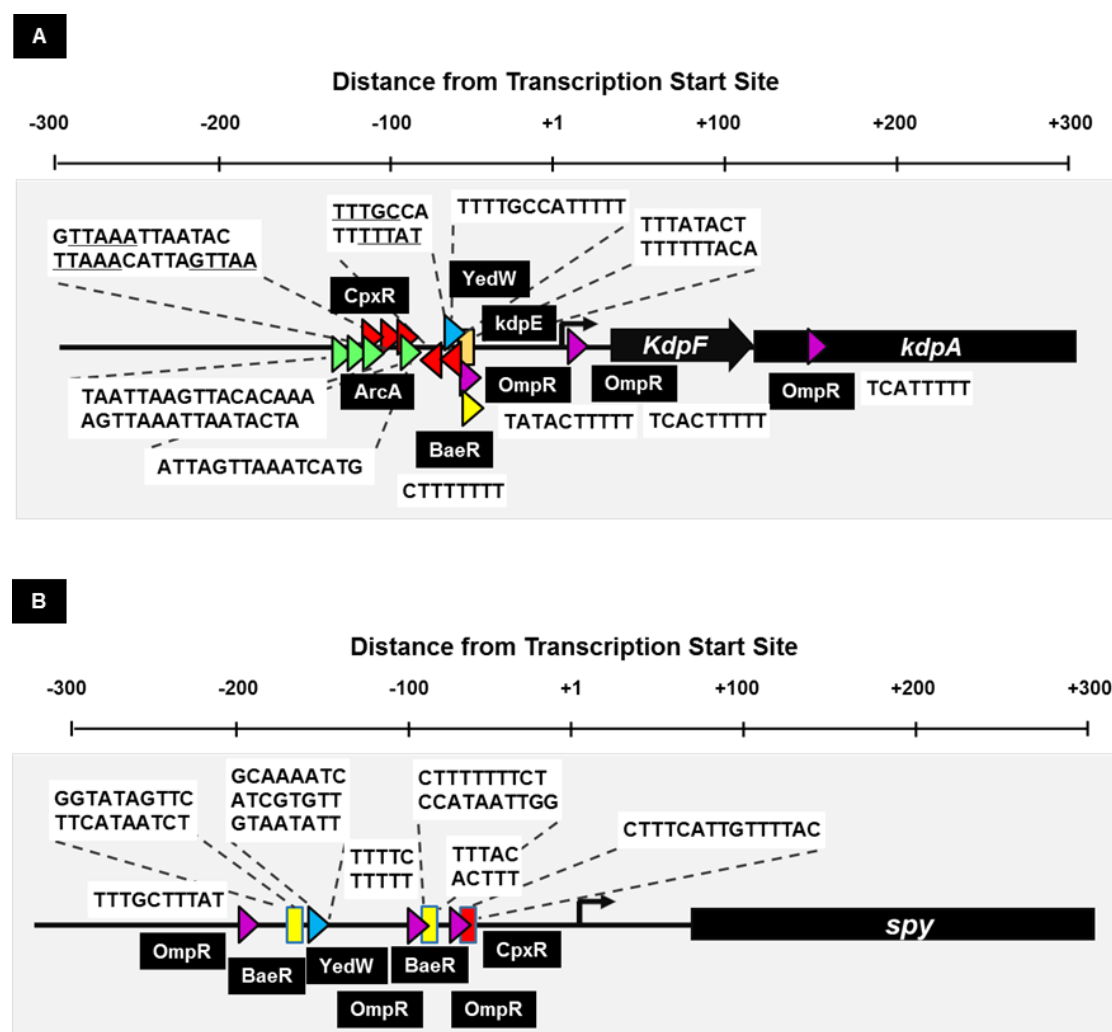


Fig 4. Location of TF-binding sites on the *kdpA* (A) and *spy* (B) promoter.

CHAPTER4

FUNCTION UNKNOWN RESPONSE REGULATOR YGEK

4-1. Introduction

E. coli possess function unknown orphan RRs. YgeK, one of these RRs, belongs to NarL-family according to HTH domain homology. And its function and cognate SK are not revealed. Previous *in vitro* study shows that *E. coli* K-12 YgeK is phosphorylated by TCS SKs BarA and UhpB, although K-12 YgeK dose not possess the receiver domain (Yamamoto *et al.* 2005). Therefore, K-12 YgeK considered that be phosphorylated by different mechanism from typical RRs.

BarA and UhpB regulates carbon metabolism respectively in *E. coli*. UhpB responds to Glucose-6-Phosphate, then UhpB phosphorylates cognate RR UhpA. Phosphorylated UhpA activates expression of *uhpT* which codes G6P transporter. In regard to BarA, it had been reported that repress the CsrA activity which activates pathogenesis, motility, quorum sensing and represses biofilm formation through the phosphorylation of cognate RR UvrY and *csrB* expression in the various bacteria. In *E. coli*, BarA-UvrY-*csrB*-CsrA system is also involved in the regulation of glycolysis, acetate metabolism, glycogen biosynthesis/catabolism and gluconeogenesis. However, there is no report likely these in other bacteria. (Lapouge *et al.* 2008). In this study, for the elucidation of the entire signal transduction network of *E. coli*, I analyzed the function of YgeK and its relation to the SK BarA and UhpB.

4-2. Materials and Methods

4-2-1. *E. coli* strains and growth conditions

E. coli W3110 type-A and *E. coli* O157:H7 Sakai was used for preparation of the expression clones of YgeK. *E. coli* BW25113 was used for monitor of Cell growth. Cells were cultured in LB medium, M9-0.4% glucose, M9-0.4% glycerol medium or M9-acetate supplemented with or without 0.2% Casamino acid at 37°C or 28°C. When necessary, 20 µg/mL chloramphenicol was added into the medium. Cell growth was monitored by measuring the optical density at 600 nm. To test biofilm formation, bacteria were inoculated on LB, M9-0.4% glycerol medium or M9-acetate agar plates containing 1.2% wt/vol of Agarose and 0.004% wt/vol of Congo red (Wako).

4-3. Results

4-3-1. Difference of YgeK between *E. coli* subspecies

For the investigate the YgeK conservation among the bacteria species, available DNA sequences which has homology to *E. coli* K-12 *ygeK* sequences were downloaded from the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (www.genome.jp/kegg). *ygeK* revealed that conserved only in *Escherichia*

genus. Furthermore, YgeK is conserved for two types: full length such as O157:H7 Sakai YgeK and short length which has not receiver domain such as K-12 YgeK (Fig. 1A, B). Full length YgeK is conserved in pathogenic *Escherichia* genus, *Escherichia albertii*, human and bird enteric pathogen, isolated from a stool sample from a 1-year-old child with acute diarrhea, *Escherichia coli* O44:H18 042, prototypical member of the enteroaggregative *E. coli* (EAEC) which shows multidrug resistant, isolated from a child with diarrhea in the course of an epidemiologic study in Lima, Peru in 1983, *E. coli* O7:K1 CE10 isolated from the cerebrospinal fluid (CSF) of a neonate with meningitis, *E. coli* O17:K52:H18 UMN026, extraintestinal pathogenic *E. coli* (ExPEC), isolated from a woman with uncomplicated acute cystitis in 1999 in the USA, *E. coli* O157:H7 Sakai, enterohemorrhagic *E. coli* (EHEC), isolated from the 1996 outbreak in primary schools of Sakai, Japan, *E. coli* O157:H7 EC4115 (EHEC) collected at the time of the 2006 spinach outbreak, *E. coli* O157:H7 TW14359 (EHEC) isolated from a patient of 2006 spinach-associated outbreak in the USA and *E. coli* O157:H7 Xuzhou21 (EHEC) isolated from an hemolytic uremic syndrome patient from the 1999 Xuzhou outbreak (Referred to KEGG) (Fig. 1A). On the other hand, short length YgeK is conserved in both pathogenic and non-pathogenic *E. coli*, O157:H7 EDL933 (EHEC) isolated from Michigan ground beef linked to the outbreak in 1982 involving contaminated hamburgers, O26:H11 11368 (EHEC) isolated in Japan in 2001 from a patient with diarrhea during a diffuse outbreak, O111:H-11128 (EHEC) isolated in Japan in 2001 from a patient with a sporadic case of diarrhea, O103:H2 12009 (EHEC) isolated in Japan in 2001 from a patient with a sporadic case of bloody stool, O104:H4 2009EL-2071 (EHEC)/O104:H4 2009EL-2050 (EHEC), shows multidrug-resistant, isolated from stool of patients in the Republic of Georgia, O104:H4 2011C-3493 (EHEC), shows multidrug-resistant, isolated from stool of a US patient with a history of travel to Germany in May 2011, E24377A, enterotoxigenic *E. coli* (ETEC), isolate from Maj. Carl Brinkley (Walter Reed Army Institute of Research), O78:H11:K80 H10407 (ETEC) isolated from an adult with cholera-like symptoms in the course of an epidemiologic study in Dacca, Bangladesh, prior to 1973, 55989 (EAEC) isolated from the diarrheagenic stools of an HIV-positive adult suffering from persistent watery diarrhea in Central African Republic, APEC O78 isolated from the lung of a turkey clinically diagnosed with colibacillosis, P12b (O15:H17) used in laboratory as a wild-type strain, O9 HS/ O152:H28 SE11/ O8 IAI1 isolated from a healthy human, UMNK88 isolated in 2007 from a farm in Minnesota, W (ATCC8739, KO11FL, LY180) isolated from the soil of a cemetery near Rutgers University around 1943 and K-12 (W3110, MG1655, MDS42, DH10B, DH1), laboratory strain (Referred to KEGG) (Fig. 1A).

In almost of short length *ygeK* gene, mutation at the Cytosin 154 to the Thymine results in the substitution of the 52th Gln to the Stop codon except for K-12 MDS42 *ygeK* which first half of receiver domain coding sequence is completely missing (Fig. 1B). This site is followed by SD-like sequence (AGGAGA) and short length YgeK is translated from Met 64 which just says in full length YgeK (Fig. 1C). Full length YgeK has the receiver domain, however not has the phosphor-Asp residue. This residue is replaced to Gly (Fig. 1B). In addition to this site, two other acidic amino acids (Asp/Glu) within the $\beta 1$ - $\alpha 1$ loop which construct the phosphate-accepting active pocket structure with the phosphor-Asp residue are Asp and Gln in YgeK, while an invariant Lys residue at the end of the $\beta 5$ strand that helps coordinate the typical active pocket is conserved in YgeK. A highly conserved Thr/Ser residue at the end of $\beta 4$ strand and a highly conserved Phe/Tyr residue

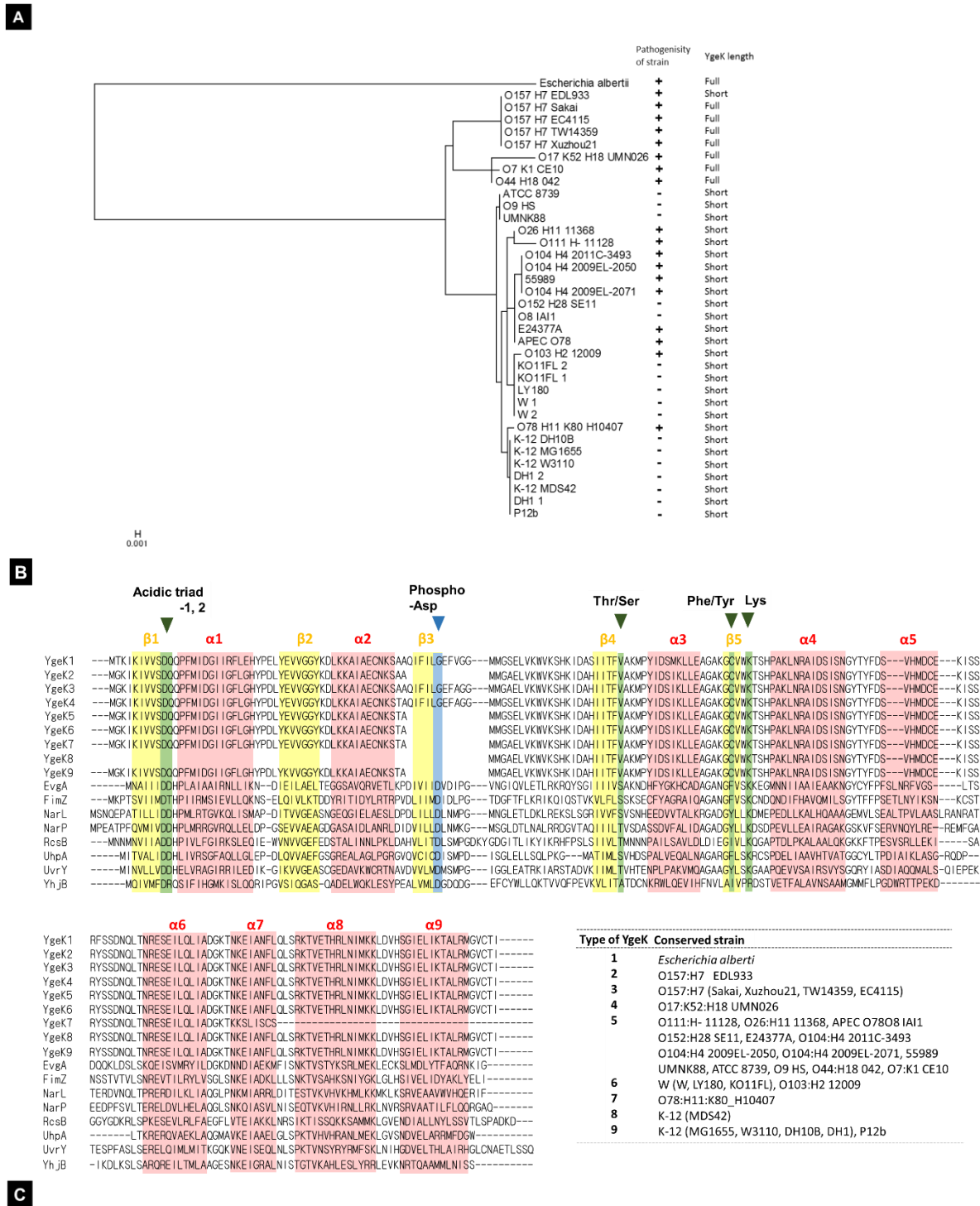


Fig. 1. YgeK conserved among *Escherichia* species. A) ygeK sequence homology between *Escherichia* species. B) The conservation of important residue for RRs activation on YgeK and other NarL-family RRs. C) Full length ygeK sequence. The bold letters indicates start codon. Under line shows SD-like sequence. 154C (shown with red) is replaced to T in the short length ygeK coding sequence.

in the middle of $\beta 5$ strand which are reorientated and able to the formation of active dimer respond to the phosphorylation of Asp residue are substituted to Val and Cys. The conserved Lys, Val and Cys in full length YgeK are also conserved in short length YgeK (Fig. 1B).

Receiver domain which does not possess phospho-Asp residue is called to Aspartate-Less Receivers (ALRs) (Maule *et al.* 2015). A residue conserved in ALR has low-like characteristics. All of three acidic residue which construct the active pocket structure is not hydrophobic residue. Conserved phospho-Asp is generally replaced to Glu, Asn, Ser, Gly and Ala which occupies 26%, 16%, 15%, 11% and 9% of entire ALRs respectively (in full length YgeK it is coordinate Gly). One of other acidic residue (acidic triad-1) is retained in 65% of ALRs, whereas the acidic triad-2 is retained only in 29% of ALRs (in full length YgeK these are Asp and Gln). The well conserved Lys, Thr/Ser residue at the end of $\beta 4$ and Phe/Tyr residue in the middle of $\beta 5$ are largely conserved in ALRs (70%, 66% and 61% respectively, in YgeK these are Lys, Val and Cys). In the ALR which is not conserved the Thr/Ser and Phe/Tyr, these residue are almost replaced to hydrophobic residue (Val, Leu, Ile and Ala) but not to a residue has a charge (Maule *et al.* 2015). Therefore, structure of full length YgeK is correspond to that of ALR. Most of ALR is distributed to the bacteria which live in extreme environment and has a pathogenicity (Maule *et al.* 2015). Full length YgeK might relate to pathogenicity of *Escherichia* genus and has a different function from short length YgeK which is conserved in non-pathogenic *E. coli* such as K-12 strain.

4-3-2. Influence of YgeK expression on the biofilm formation of *E. coli*

The sequence comparison described above revealed that YgeK homologs are roughly divided into two types: full-length and short-length. To ask whether the two types of ygeK have different functions, the coding sequences of the ygeK genes of O157: H7 Sakai (full-length) and K-12 W3110 type A (short-length) were PCR amplified from their genomes. Into the 5'-primer sequence, the typical ribosome recognition sequence (SD sequence) was added. In addition, the 3'-primers included a FLAG-tag sequence so as to be expressed as fusion with RRs. The PCR-amplified FLAG-tagged YgeK-coding sequences were inserted into *Sac* I and *Hind*III treated pBAD33 to construct the arabinose-inducible expression plasmids of FLAG-tagged YgeK. Using these plasmids, *E. coli* K-12 BW25113 strain were transformed and cultured with 0.02% arabinose for induction of YgeK expression. Sakai YgeK expressed cells in the M9-Glycerol supplemented with Casamino acid at 37°C sank in the bottom of test tube and seems to adhere to the test tube when the culture was left at rest for a while (Fig. 2A). It is considered that fimbriae synthesis was activated in the Sakai YgeK expressed cells.

Biofilm (cellulose, other related polysaccharides, fimbriae) has a high affinity to Congo red dye (Olsen *et al.* 2002). If biofilm is formed, bacterial colony observed to red on the Congo red agar. To confirm whether Sakai YgeK expressed cells forms biofilm or not, WT, ygeK deletion mutant and K-12 YgeK or Sakai YgeK expressed ygeK deletion mutant inoculated on the Congo red agar plate (M9-Glycerol supplemented Casamino acid and 0.02% Arabinose agar plate) and color of colony formed at 37°C was observed (Fig. 2B). WT and ygeK deletion mutant formed orange colony. When K-12 YgeK was expressed in the ygeK deletion mutant colony color became light, while Sakai YgeK expression result in red and rough colony formation. It

suggest that both of K-12 YgeK and Sakai YgeK play the role of biofilm formation, however Sakai YgeK activates and K-12 YgeK represses that at 37°C.

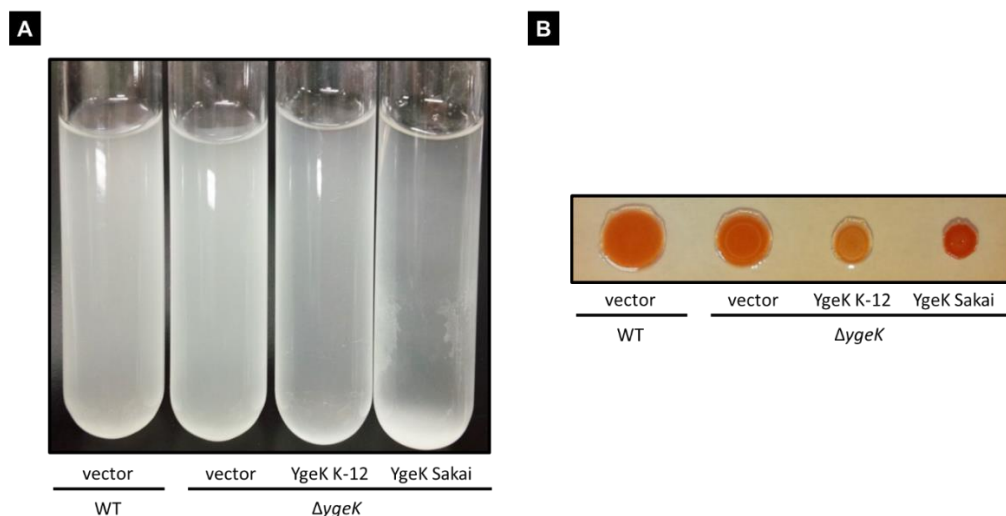


Fig. 2. YgeK function for Biofilm formation. *E. coli* K-12 BW25113 strain (WT), *ygeK* deletion mutant ($\Delta ygeK$) and $\Delta ygeK$ transformed by K-12 *ygeK* expression plasmid (*pygeK* K-12) or O157:H7 sakai *ygeK* expression plasmid (*pygeK* sakai) were cultured in 0.2% Casamino acid and 0.02% Arabinose supplemented M9-0.4%Glycerol (A), and inoculated on the 0.004% Congo red, 0.2% Casamino acid and 0.02% Arabinose supplemented M9-0.4%Glycerol agar plate (B).

4-3-3. Factor influence on YgeK function

Previous study shows that YgeK is phosphorylated by BarA and UhpB *in vitro* (Yamamoto *et al.* 2005). To examine whether BarA and UhpB participate in the YgeK function *in vivo*, YgeK was expressed in a *barA*- or *uhpB*-deletion derivative of the standard K12 strain BW25113 and the biofilm formation of the resulting strains was observed. However, the expression of Sakai YgeK in either a *barA*- or *uhpB*-deletion mutant did not significantly affect the colony color regardless of the presence of the chromosomal *ygeK* gene (Fig. 3B). It should be noted that the activity of an RR is regulated not only through phosphotransfer from a phosphorylated SK but also through direct phosphorylation by acetylphosphate (AcP) and through acetylation using acetyl-CoA (Fig. 3A). Thus, the effect of the YgeK expression on biofilm formation of mutants lacking *ackA* (acetate kinase A), *pta* (phosphotransacetylase), *acs* (acetyl-CoA synthetase), *yfiQ* (protein lysine acetyltransferase) or *cobB* (deacetylase of Acs and CheY) were observed. The expression of Sakai YgeK did not significantly affect the colony color or the colony morphology of these deletion mutants. These results indicate that biofilm formation function of Sakai YgeK is independent on SKs (BarA and UhpB), acetyl phosphate, the acetyltransferase and the deacetylase.

Formation of Mat fimbria, highly conserved between different origin *E. coli* and considered to be an ancestral fimbrial type in *E. coli*, is regulated by temperature (Lehti *et al.* 2010). To confirm whether YgeK function of biofilm formation is also regulated by temperature, WT, *ygeK* deletion mutant and K-12 YgeK or Sakai YgeK expressed *ygeK* deletion mutant were inoculated on the Congo red agar plate (M9-Glycerol supplemented Casamino acid and 0.02% Arabinose agar plate) and incubate at 28°C (Fig. 3C). K-12 YgeK expression at 28°C didn't results in light color colony formation, different from 37°C. Sakai YgeK expressed

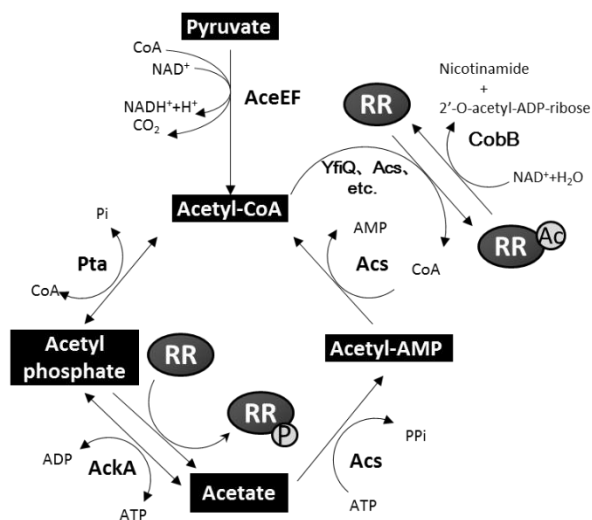
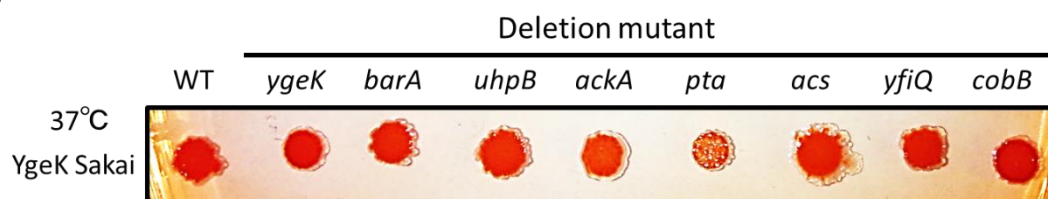
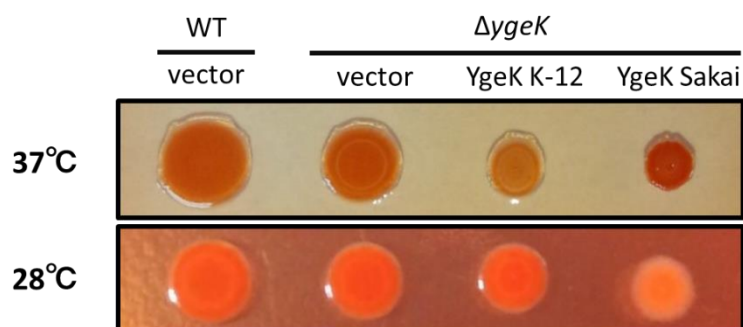
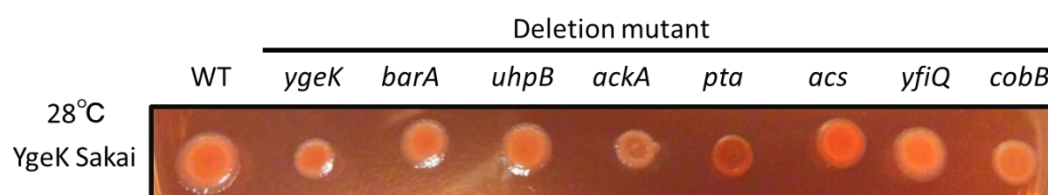
A**B****C****D**

Fig. 3. Factor influence on YgeK biofilm formation function. *E. coli* K-12 BW25113 strain (WT), *ygeK*, *barA*, *uhpB*, *ackA*, *pta*, *acs*, *yfiQ* and *cobB* deletion mutant transformed by pBAD33, K-12 *ygeK* expression plasmid (pygeK K-12) or O157:H7 sakai *ygeK* expression plasmid (pygeK sakai) were inoculated on the 0.004% Congo red, 0.2% Casamino acid and 0.02% Arabinose supplemented M9-0.4% Glycerol agar plate. These were incubated at 37°C or 28°C.

cell formed a white and slight rough colony at 28°C, while it formed a red and rough colony at 37°C (Fig. 3C). It is indicated that a function of YgeK of both K-12 and Sakai are regulated by temperature. Moreover, the fact that Sakai YgeK expressed cell forms white and rough colony at 28°C suggests repression of extracellular polysaccharide synthesis in this cell. This function of Sakai YgeK at 28°C is not observed in *pta* or *acs* deletion mutant, while observed in *barA*, *uhpB*, *ackA*, *yfiQ* and *cobB* deletion mutant (Fig. 3D). It is indicated that that is depends on Pta and Acs which are needed for acetyl-CoA synthesis (Fig. 3A).

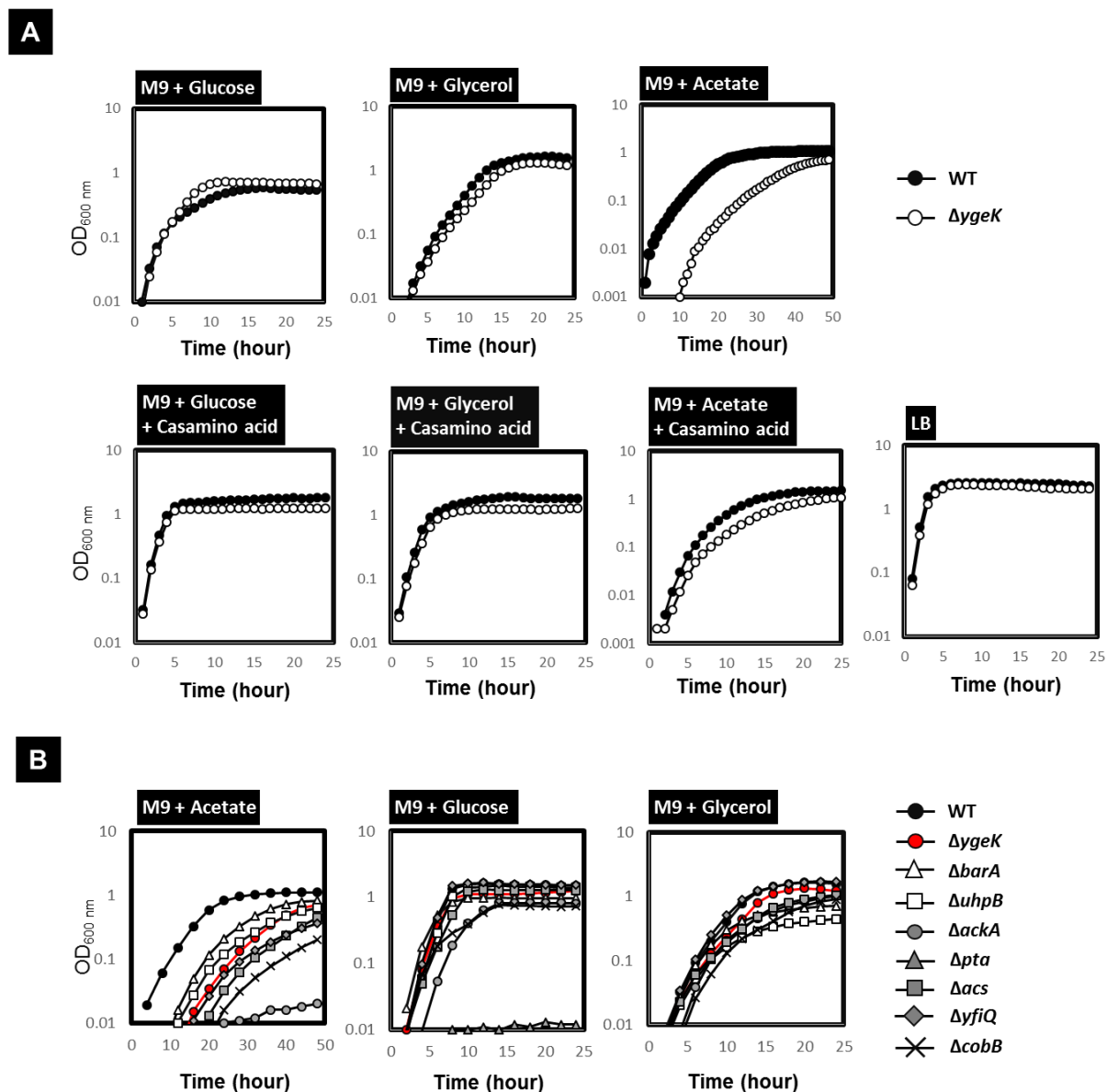


Fig. 4. Growth of *ygeK* deletion mutant. *E. coli* K-12 BW25113 strain (WT), *ygeK*, *barA*, *uhpB*, *ackA*, *pta*, *acs*, *yfiQ* and *cobB* deletion mutant ($\Delta ygeK$, $\Delta barA$, $\Delta uhpB$, $\Delta ackA$, Δpta , Δacs , $\Delta yfiQ$ and $\Delta cobB$ respectively) were cultured in M9-0.4% Glucose medium over night at 37°C. Over night culture were diluted 100 times with M9-0.4% Glucose, M9-0.4% Glycerol, M9-50 mM Acetate with or with 0.2% Casamino acid or LB medium followed by measuring OD 600 nm at 37 C° with shaking.

4-3-4. Growth retardation in acetate medium

YgeK has a possibility that involved in the carbon metabolism through the BarA, UhpB and the pyruvate-acetate pathway. Therefore growth of *ygeK* deletion *E. coli* mutant was observed in different nutrient medium (Fig. 4A). Growth of $\Delta ygeK$ mutant in acetate medium without casamino acid was retarded compared to that of WT *E. coli* strain, while in LB medium, Glucose medium, Glycerol medium and casamino acid supplemented medium, $\Delta ygeK$ mutant grew up as well as WT. These results suggest that YgeK plays the important role for the *E. coli* K-12 growth at the acetate medium.

Deletion of AckA or Pta, main pathway of acetate metabolism, result to fails to grow in the acetate medium. Not only that, *pta* deletion mutant is unable to grow in other minimum medium without casamino acid (Fig. 4B). On the other hand, deletion of Acs, another pathway of acetate metabolism, showed growth retardation in acetate medium similar to YgeK deletion (Fig. 4B). In addition, deletion of acetyltransferase YfiQ and deacetylase CobB also showed growth retardation in acetate medium. Acetylation and deacetylation are indicated that also participate to acetate metabolism. Growth of $\Delta barA$ and $\Delta uhpB$ mutant were also retarded (Fig. 4B). This growth retardation of $\Delta barA$ and $\Delta uhpB$ mutant is only observed in acetate medium, although decreased growth of $\Delta barA$ and $\Delta uhpB$ mutant were observed in the glycerol medium. YgeK activity for *E. coli* growth in acetate medium might be controlled by BarA and UhpB, and those are act on *E. coli* acetate metabolism together with the pyruvate-acetate pathway.

4-4. Discussion

4-4-1. Difference of YgeK function between K-12 and O157:H7 Sakai

Most of ALR is distributed to the bacteria which live in extreme environment and has a pathogenicity (Maule *et al.* 2015). Full length YgeK correspond to ALR is conserved in pathogenic *E. coli* such as O157:H7 strain, although in the experimental *E. coli* strain, short length YgeK is conserved. Bacterial adherence is known to be important so that bacteria exhibit pathogenicity. Full length YgeK has an ability of biofilm formation at 37°C. Therefore full length YgeK might be related to the *E. coli* adherence to the host.

Short length YgeK which does not possess receiver domain is also related to biofilm formation. Furthermore, it plays the role for the *E. coli* K-12 growth at the acetate medium. YgeK possibly maintains the necessary function while changing its form and function adapt to environment.

4-4-2. Signal transduction of YgeK

Growth of *ygeK* deletion mutant was retarded in acetate medium compared to that of WT. In the same way, deletion of *barA* or *uhpB* result to growth retardation in acetate medium. Previous *in vitro* study shows that K-12 YgeK is phosphorylated by BarA and UhpB. BarA-UvrY regulates various cell processes including biofilm formation and acetate metabolism. UhpBA also participate to metabolism through the transport of Glucose-6-phosphate. In addition, YgeK also regulates biofilm formation. Sakai YgeK function to activate biofilm formation is regulated by temperature. Moreover, biofilm repression function of Sakai YgeK is dependent on *pta* and *acs*. In the acetate medium, growth of K-12 deletion mutant of *acs*, *yfiQ*, *cobB* is retarded

similar to that of *ygeK*. Acetate metabolism function of YgeK, perhaps, related to its acetylation. In any case, it is indicated that YgeK is regulated by temperature and metabolism and that YgeK is pathway links the metabolism and the gene expression.

In this study, YgeK phenotype were observed. However further analysis of this mechanism is necessary. At present, SELEX analysis for identification of YgeK recognition target promoter is going.

CHAPTER 5

CONCLUSIONS

5-1. Specific and various signal transduction network of TCS

Analysis of the stage 3 cross talk between NarL-family RRs described in the chapter 2 revealed that stage 3 cross talk occurs in limited combinations and those seem to be specific. In the chapter 3, the stage 3 cross talk between OmpR-family RRs also observed and it seems to be specific and precise event to enable TCS signal transduction complicated. Furthermore, the *ydeP* promoter, the *spy* promoter and the *kdpA* promoter revealed to multifactor promoters. These genes might play important role for *E. coli* adaptation to the various environmental changes.

Under *in vivo* situations, SK controlled RR activity by phosphorylation in generally, while a set of RRs is also activated directly by AcP but independent of SK-RR pathway (McCleary & Stock 1994; Pruss & Wolfe 1994). The intracellular concentration of AcP is strongly dependent on the metabolic state of the cell, as well as on growth phase, carbon source, pH, and temperature (McCleary & Stock 1994; Pruss & Wolfe 1994). Therefore, the concentration of cytoplasmic AcP can be regarded as a physiologically relevant signal, feeding into the signal transduction systems of *E. coli*. In addition to AcP, there is a several report that acetylation of RR act on the RRs activity (Barak & Eisenbach, 1996; Thao *et al.* 2010). Acetylation is also presumed to strongly dependent on the metabolic state of the cell, as well as on growth phase, carbon source, pH, and temperature. The stage 3 cross talk may be especially important as a way of directly linking different regulation systems in a network to coordinate cell growth and metabolism.

Not only these results but also analysis of YgeK function, described in chapter 4, revealed that atypical RR which does not possess the phospho-Asp or the receiver domain also plays an important role in *E. coli*. Short length K-12 YgeK has a function for *E. coli* growth in acetate medium and it has also acts on repression of biofilm formation at 37°C. On the other hand, full length O157: H7 Sakai YgeK activate a biofilm formation at 37°C while repress at 28 °C. Those YgeK function seems to be related to acetylation. YgeK is also considered that link the cell growth or metabolism to the gene regulation.

Taking all the results together, these findings provide the insight into the perspective of TCS signal transduction network and contribute for understanding the mechanism of the bacterial adaptation and survival against to environment change.

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LIST OF PUBLICATIONS

1. Kinoshita, E., Kinoshita-Kikuta, E., Shiba, A., Edahiro, K., Inoue, Y., Yamamoto, K., Yoshida, M. & Koike, T. (2014)
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