Full Paper

Fe³⁺-dependent epistasis between the CpxR-activated loci and the PmrA-activated LPS modification loci in *Salmonella enterica*

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Bacteria utilize varying combinations of two-component regulatory systems, many of which respond and adapt closely to stress conditions, thus expanding their niche steadily. While mechanisms of recognition and avoidance of the specific Fe³⁺ signal by the PmrA/PmrB system is well understood, those of the CpxR/CpxA system are more complex because they can be induced by various stress conditions, which, in turn, expresses a variety of phenotypes. Here, we highlight another aspect of the CpxR/CpxA system; mutations in *degP* and *yqjA* genes, which are under the control of the system, exhibit an iron sensitive phenotype in the mutant background defective in the PmrA-dependent gene products that alter the pyrophosphate status of the lipid A moiety of lipopolysaccharide in *Salmonella enterica*. Therefore, after the PmrA/PmrB-mediated Fe³⁺-dependent control of the pyrophosphate status on the cell surface, the CpxR/CpxA system is one of the second layers of envelope stress response that allows adaptation to high Fe³⁺ conditions in this bacterium.

Key Words: Cpx; genetic interaction; lipopolysaccharide (LPS); metal; PmrR; reactive oxygen species (ROS); *Salmonella enterica*; two-component system

Introduction

The PmrA/PmrB two-component regulatory system confers *Salmonella* resistance to cationic peptide polymyxin B and ferric iron by modifying the phosphoryl groups of lipopolysaccharide (LPS) (Chen and Groisman, 2013), the

major component of the outer membrane of Gram-negative bacteria. The regulator protein PmrA activation status responds not only to extracellular Fe³⁺, a signal of the sensor histidine kinase PmrB (Wösten et al., 2000), but also to low Mg²⁺, an activation signal of another two-component regulatory system PhoP/PhoQ (García Vescovi et al., 1996), in a PmrD-dependent manner (Kato and Groisman, 2004; Kox et al., 2000). Thus, under high Fe³⁺, or low Mg²⁺, conditions, the PmrA/PmrB system activates genes such as *ugd/pbgPE*, *pmrC*, and *pmrR*, which together change the chemical structure of the lipid A anchor of LPS (Fig. 1). Ugd/PbgPE modifies phosphates of lipid A with L-4-aminoarabinose (L-Ara4N) at the 1 and 4' positions (Gunn et al., 1998), whereas PmrC adds phosphoethanolamine (pEtN) primarily to phosphate at the 1 position of lipid A (Kato et al., 2012; Lee et al., 2004). The PmrC-mediated pEtN modification at position 1 of lipid A requires the concomitant action of a novel membrane peptide PmrR (Kato et al., 2012), which inhibits the formation of the LpxT-mediated pyrophosphate at position 1 (1-PP) (Herrera et al., 2010; Touze et al., 2008). In addition, in the presence of both high Fe³⁺, and low Mg²⁺, signals, phospho-PmrA binds to the *pmrD* promoter to exert partial inhibition of PhoP-dependent transcriptional initiation (Kato et al., 2003). Previous studies have demonstrated that the iron sensitivity of the *pmrAB* mutant was equivalent to that of a triple mutant at loci encoding for the PmrA-activated proteins, Ugd/PbgPE, PmrC, and PmrG, the last of which is a phosphatase that targets the Hep(II) phosphate in the core region of the LPS (Nishino et al., 2006), while polymyxin B sensitivity of the *pmrA* mutant was equivalent to that of a double mutant at loci encoding for the PmrA-activated Ugd/PbgPE and PmrC proteins (Lee et al., 2004). One of the above three pathways was sufficient for exhibiting wild-type level iron resistance on solid media (Nishino et al., 2006).

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Fig. 1. Schematic drawing structures of the lipid A moiety of lipopolysaccharide (LPS) and of the lipid A modifications in *Salmonella*.

The predominant lipid A species is hexa-acylated and phosphorylated at the 1 and 4' positions. LpxT adds a second phosphate group to the 1-position resulting in a 1-pyrophosphorylated (1-PP) species. While the lipid A phosphates can also be modified with L-4-aminoarabinose (L-Ara4N) by Ugd and PbgPE, a PmrC-mediated phosphoethanolamine (pEtN) modification can occur rather preferentially at the 1-position of the lipid A phosphate in inducing conditions of the PmrA/PmrB system (i.e., low Mg^{2+} and high Fe^{3+}). The LpxT-dependent 1-PP modification is predominant (>) over the PmrC-dependent 1-pEtN modification (Herrera et al., 2010), unless PmrR inhibits LpxT (Kato et al., 2012).

Our group has reported the reciprocal control of the PmrA/PmrB system and the LPS modification status where, together with the Ugd/PbgPE-mediated positively charged L-Ara4N modification at the 1 and 4' positions of lipid A, the PmrC-mediated pEtN modification at the 1 position and the PmrR dependent inhibition of the 1-PP lipid A formation, which is catalyzed by LpxT, decreases the net negative charge of the LPS and, in turn, hinders the accessibility of Fe^{3+} to the cell surface as well as to the Fe^{3+} sensor PmrB (Kato et al., 2012). Moreover, we have demonstrated that the formation of 1-PP lipid A is essential to the reciprocal control phenomenon and iron binding to the cell surface because they are completely abolished by mutating the *lpxT* gene (Kato et al., 2012). Additionally, while attempting to identify the PmrR peptide target, a pull-down with GST-PmrR resulted in the identification of several proteins, including DegP that is under the control of the CpxR/CpxA system in *E. coli* and *S. enterica*, as co-purified proteins (Kato, A., unpubl.). Thus, we have assumed possible relationships between the PmrA/PmrB and CpxR/CpxA systems under high ferric iron conditions.

The CpxR/CpxA system is responsive to envelope stress, such as misfolded proteins, alkaline pH, altered membrane phospholipid composition, perturbation in lipoprotein composition, adhesion, indole, ethanol, copper, EDTA, and high osmolality, in many gamma proteobacteria, including *E. coli* and *S. enterica* (Raivio, 2014). The CpxR/CpxA system is required for antibiotic resistance, including aminoglycosides, novobiocin, and β -lactams, survival in deoxycholate (Hirakawa et al., 2003a, b) and cationic antimicrobial peptides, such as protamine, magainin 2, and melitin (Shi et al., 2004; Weatherspoon-Griffin et al., 2011), as well as copper resistance (Yamamoto and Ishihama, 2006). Some of the sensitive phenotypes observed in the Cpx mutants in the presence of these agents

are, at least in part, likely due to changes in the outer membrane permeability (Raivio, 2014), as a deficiency in the CpxR-activated DegP protease activity resulted in altered levels in the outer membrane proteins (OMPs) OmpC, PmpF, OmpW, OmpX, and OmpA, as well as FkpA, a key quality control factor for the biogenesis of outer membrane proteins in *E. coli* (Ge et al., 2014). The CpxR/CpxA-induced cell wall amidases AmiA and AmiC elevated cationic antimicrobial peptide resistance in *S. enterica* (Weatherspoon-Griffin et al., 2011). Activation of the CpxR/CpxA system leads to a decrease or alteration in the proton motive force (PMF), which would, in turn, increase antibiotic resistance (Raivio, 2014). It has been suggested that the aminoglycoside resistance phenotype is caused by decreased levels of PMF-dependent uptake in *E. coli* (Ezraty et al., 2013). PMF is essential for efflux systems, including the CpxR-activated AcrD as well as MdtABC RND efflux pumps. However, currently, explicit links between sources of altered PMF and its related phenotypes upon Cpx induction or its mutations are not well understood. On the one hand, many gene products, such as Sdh, Nuo, Cyo, NhaB, DctA, and TppB, which may contribute to PMF generation, are downregulated by CpxR/CpxA at the transcriptional or post-transcriptional levels in *E. coli* (Raivio, 2014). On the other hand, for a possible PMF contribution, at least transcription of *yqjA* and *yceJ*, which encodes a putative osmosensing transporter that is required for growth at alkaline pH (Kumar and Doerrler, 2015) and a putative cytochrome (Raivio et al., 2013), respectively, is upregulated by the CpxR/CpxA system in *E. coli*. In this report, we have tested the effects of the *cpxR*, *degP*, and *yqjA* mutations on *Salmonella* cell growth in the presence of high Fe^{3+} in mutant backgrounds defective in the Ugd-mediated positively charged L-Ara4N modification at the 1 and 4' positions of lipid A, as well as the action of the PmrR peptide that inhibits the 1-PP lipid A synthetase LpxT.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. All *Salmonella enterica* serovar Typhimurium strains are derived from wild-type 14028s and were constructed by phage P22-mediated transductions as described (Davis et al., 1980). Bacteria were grown at 37°C in an N-minimal media (Snavelly et al., 1991) buffered with 50 mM Bis-Tris, pH 7.7, and supplemented with 0.1% casamino acids, 38 mM glycerol and 10 μ M or 10 mM $MgCl_2$. Ampicillin and kanamycin were used at 50 μ g/ml, chloramphenicol at 20 μ g/ml, and gentamicin at 10 μ g/ml. Primers used in this study are listed in Table 2.

Construction of chromosomal gene deletion mutants. Strain AK1570, which has a deletion of the *yqjA* gene and a Cm^R cassette, was constructed by the one-step inactivation method (Datsenko and Wanner, 2000) using primers A209 and A210 with pKD3 as the template.

Strain AK1571, which has a deletion of the *copR* gene and a Cm^R cassette, was constructed by the same method using primers A250 and A251 with pKD3 as the template.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
<i>S. enterica</i>		
14028s	Wild type	ATCC
EG9524	<i>ugd::MudJ</i>	García Vescovi et al. (1996)
EG15437	<i>pmrR_{promoter}</i>	Kato et al. (2012)
EG17203	<i>pmrR_{promoter} ugd::MudJ</i>	Kato et al. (2012)
AK1007	<i>ΔcpxR::Cm^R</i>	Kato et al. (2012)
AK1010	<i>ΔdegP::Cm^R</i>	Kato et al. (2012)
AK1011	<i>ΔdegP</i>	This work
AK1012	<i>pmrR_{promoter} ΔdegP::Cm^R</i>	This work
AK1013	<i>pmrR_{promoter} ΔdegP</i>	This work
AK1014	<i>ugd::MudJ ΔdegP::Cm^R</i>	This work
AK1015	<i>ugd::MudJ ΔdegP</i>	This work
AK1016	<i>pmrR_{promoter} ugd::MudJ ΔdegP::Cm^R</i>	This work
AK1017	<i>pmrR_{promoter} ugd::MudJ ΔdegP</i>	This work
AK1018	<i>pmrR_{promoter} ΔcpxR::Cm^R</i>	This work
AK1020	<i>ugd::MudJ ΔcpxR::Cm^R</i>	This work
AK1022	<i>pmrR_{promoter} ugd::MudJ ΔcpxR::Cm^R</i>	This work
AK1024	<i>ΔdegP ΔcpxR::Cm^R</i>	This work
AK1026	<i>pmrR_{promoter} ΔdegP ΔcpxR::Cm^R</i>	This work
AK1028	<i>ugd::MudJ ΔdegP ΔcpxR::Cm^R</i>	Kato et al. (2012)
AK1029	<i>ugd::MudJ ΔdegP ΔcpxR</i>	Kato et al. (2012)
AK1030	<i>pmrR_{promoter} ugd::MudJ ΔdegP ΔcpxR::Cm^R</i>	Kato et al. (2012)
AK1031	<i>pmrR_{promoter} ugd::MudJ ΔdegP ΔcpxR</i>	Kato et al. (2012)
AK1032	<i>ΔlpxT::Cm^R</i>	Kato et al. (2012)
AK1036	<i>ugd::MudJ ΔdegP ΔcpxR ΔlpxT::Cm^R</i>	Kato et al. (2012)
AK1037	<i>pmrR_{promoter} ugd::MudJ ΔdegP ΔcpxR ΔlpxT::Cm^R</i>	Kato et al. (2012)
AK1038	<i>pmrR_{promoter} ΔlpxT::Cm^R</i>	Kato et al. (2012)
AK1040	<i>ugd::MudJ ΔlpxT::Cm^R</i>	Kato et al. (2012)
AK1041	<i>pmrR_{promoter} ugd::MudJ ΔlpxT::Cm^R</i>	Kato et al. (2012)
AK1570	<i>ΔyqjA::Cm^R</i>	This work
AK1571	<i>ΔcopR::Cm^R</i>	This work
AK1572	<i>ΔdegP ΔyqjA::Cm^R</i>	This work
AK1573	<i>ΔdegP ΔcopR::Cm^R</i>	This work
AK1574	<i>pmrR_{promoter} ΔyqjA::Cm^R</i>	This work
AK1575	<i>pmrR_{promoter} ΔcopR::Cm^R</i>	This work
AK1576	<i>pmrR_{promoter} ΔdegP ΔyqjA::Cm^R</i>	This work
AK1577	<i>pmrR_{promoter} ΔdegP ΔcopR::Cm^R</i>	This work
AK1578	<i>ugd::MudJ ΔyqjA::Cm^R</i>	This work
AK1579	<i>ugd::MudJ ΔcopR::Cm^R</i>	This work
AK1580	<i>pmrR_{promoter} ugd::MudJ ΔyqjA::Cm^R</i>	This work
AK1581	<i>pmrR_{promoter} ugd::MudJ ΔcopR::Cm^R</i>	This work
AK1582	<i>ugd::MudJ ΔdegP ΔyqjA::Cm^R</i>	This work
AK1583	<i>ugd::MudJ ΔdegP ΔcopR::Cm^R</i>	This work
AK1584	<i>pmrR_{promoter} ugd::MudJ ΔdegP ΔyqjA::Cm^R</i>	This work
AK1585	<i>pmrR_{promoter} ugd::MudJ ΔdegP ΔcopR::Cm^R</i>	This work
AK1586	<i>ugd::MudJ ΔdegP ΔyqjA</i>	This work
AK1587	<i>pmrR_{promoter} ugd::MudJ ΔdegP ΔyqjA</i>	This work
AK1588	<i>ugd::MudJ ΔdegP ΔyqjA ΔlpxT::Cm^R</i>	This work
AK1589	<i>pmrR_{promoter} ugd::MudJ ΔdegP ΔyqjA ΔlpxT::Cm^R</i>	This work
AK1590	<i>ugd::MudJ ΔdegP ΔlpxT::Cm^R</i>	This work
AK1591	<i>pmrR_{promoter} ugd::MudJ ΔdegP ΔlpxT::Cm^R</i>	This work
<i>E. coli</i>		
DH5α	<i>fhuA2 lac(del)U169 phoA glnV44 80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	Hanahan (1983)
Plasmids		
pRU1097	pBBR1 ori Gm ^R <i>gfpmut3.1</i>	Karunakaran et al. (2005)
pAKlux2.1	pBBR1 ori Ap ^R <i>luxCDABE</i>	Karsi and Lawrence (2007)
pLUX1097-p _{cpxP}	pBBR1 ori Gm ^R p _{cpxP} - <i>luxCDABE</i>	This work

Plasmid construction. Plasmid pLUX1097-p_{cpxP} harboring the *cpxP* promoter in front of the *luxCDABE* reporter genes was constructed by cloning a PCR fragment amplified using primers A1085 and A1086 and 14028s genomic DNA as a template between the *SalI* and *PstI* sites of pRU1097, the *gfp* reporter plasmid (Karunakaran et al., 2005), which was followed by the

replacement of *gfp* at the *PstI* and *SacI* sites with a PCR fragment generated using primers A2412 and A2413 and DNA of pAKlux2.1, the *luxCDABE* reporter plasmid, (Karsi and Lawrence, 2007) as a template.

Cell growth analysis. A cell growth assay in a liquid media containing various concentrations of metals was performed as previously described (Kato et al., 2012). The relative

Table 2. Primers used in this study.

Primer name	Sequence
A209	ATCACCGGCGTGACGTTAACCGATTCTCCAGGAATACTGACATATGAATATCCTCCTTAG
A210	TGAGTTGTGCGGCAGTTATGCGTGGTTTAAGCATAAGTTAGTGTAGGCTGGAGCTGCTTC
A250	ATTTACGGATTGCTAACAAGCCTATGGCATACTGCGTTGGTGTAGGCTGGAGCTGCTTC
A251	TAAAAGAAATTGTCAGGCGCAGCGTCATTGATAACTTAACCATATGAATATCCTCCTTAG
A1085	ACGCGTCGACTGTTTACGTACCTCCGA
A1086	AACTGCAGC GCTTGCTCCCAAAATC
A2412	GGGGTACCAGGCTTGAGGATAC GTAT
A2413	GGAAATTCGAGCTCTCAACTATCAAACGCTTCG

Fig. 2. Growth of isogenic *pmrR*⁺ and *pmrR* promoter mutant strains with deletions in the *ugd*, *degP* or *cpxR* genes (or a combination of these genes) following growth for 12 h in an N-minimal medium at pH 7.7 with 10 μ M Mg²⁺ in the absence (A), or presence (B), of 400 μ M FeCl₃.

Bacterial growth was determined in wild-type (14028s), *pmrR* promoter mutant (EG15437), *ugd* mutant (EG9524), *ugd pmrR* promoter mutant (EG17203), $\Delta degP$ mutant (AK1011), $\Delta degP pmrR$ promoter mutant (AK1013), $\Delta degP ugd$ mutant (AK1015), $\Delta degP ugd pmrR$ promoter mutant (AK1017), $\Delta cpxR$ mutant (AK1007), $\Delta cpxR pmrR$ promoter mutant (AK1018), $\Delta cpxR ugd$ mutant (AK1020), $\Delta cpxR ugd pmrR$ promoter mutant (AK1022), $\Delta cpxR \Delta degP$ mutant (AK1024), $\Delta cpxR \Delta degP pmrR$ promoter mutant (AK1026), $\Delta cpxR \Delta degP ugd$ mutant (AK1028), and $\Delta cpxR \Delta degP ugd pmrR$ promoter mutant (AK1030) strains. Data correspond to the mean of three independent experiments performed in triplicate and error bars represent standard deviations.

growth rate was obtained by dividing the OD₅₉₅ values from each of the strains grown in the presence of various concentrations of metals, FeCl₃, CuSO₄, and ZnSO₄ by the OD₅₉₅ values from corresponding strains grown in the absence of these metals.

Luminescence assay. Bacterial cells, containing pLUX1097-*p_{cpxP}* from overnight cultures grown in an N-minimal medium at pH 7.7 with 10 μ M MgCl₂ and gentamycin, were added to 200 μ l of fresh media without gentamycin in the presence of various concentrations of metals with a 1:50 dilution in a 96-well round-bottom tissue culture test plate (TPP) and shaken at 37°C for 8 h. After the OD₅₉₅ was measured directly from the plate using a Model 680 microplate reader (BioRad), 100 μ l of culture were transferred to OptiPlate-96, White Opaque 96-well Microplate (PerkinElmer) and the luminescence

was measured by Plate CHAMELEON V (Hidex). The luminescence values were divided by the corresponding OD₅₉₅ values and multiplied by 2 to obtain a relative luminescence value. The induction ratio was obtained by dividing the relative luminescence values from wild-type and *cpxR* mutant cells harboring pLUX1097-*p_{cpxP}* grown in the presence of various concentrations of metals, FeCl₃, CuSO₄, and ZnSO₄, by that from wild-type cells pLUX1097-*p_{cpxP}* grown in the absence of these metals.

Isolation and TLC analysis of lipid A species from ³²P-labeled cell. Bacteria were grown and ³²P-labeled lipid A was isolated as described (Herrera et al., 2010) with the following modifications: ³²P-labeled lipid A species (~200 cpm per lane) were analyzed using TLC in a solvent system of chloroform, pyridine, 88% formic acid, and water (50:50:16:5, v/v), for ~3 h. The plate was exposed to a BAS-MS2040 imaging plate for several days and visualized using an FLA-7000 imaging system (Fujifilm). For the analysis of the Fe³⁺-dependent loss of the 1-PP moiety of lipid A, 100 μ M FeCl₃ was added to the LPS sample that had been prepared from wild-type cells grown in 5 ml of an N-minimal medium, pH 7.7, with 10 mM MgCl₂ (i.e., non-inducing conditions), immediately before the non-enzymatic cleavage reaction of glycoside linkage between KDO and lipid A at 100°C, in 0.45 ml of 12.5 mM sodium acetate, pH 4.5, containing 1% SDS. The ³²P-labeled lipid A species (~200 cpm per lane) were analyzed using TLC as described above.

Results and Discussion

Fe³⁺-dependent epistasis within and between regulon members

To test the genetic determinants contributing to permissive growth of *S. enterica* under high iron conditions, a cell growth analysis in a liquid N-minimal media, pH 7.7, with 10 μ M MgCl₂ and various concentrations of FeCl₃, was performed as previously described (Kato et al., 2012). A single mutation in the LPS modification gene *ugd*, but not *pmrR*, exhibited a significant impairment in cell growth under high ferric iron conditions (Figs. 2B and 3), but not in the same media without the addition of iron (Fig. 2A). However, the *pmrR* promoter mutation also became severe for cell growth under the same conditions when it was combined with the *ugd* mutant, exhibiting an epistasis in the growth inhibition phenotype in the presence of Fe³⁺ (Figs. 2B and 3), which is somewhat different from Fe³⁺ signal accessibility-dependent redundant reciprocal controls independently closed by both the *ugd* and *pmrR*

gene products, respectively (Kato et al., 2012). (Because *pmrR* is encoded closely to the 3' end of the *pmrB*-coding region in its opposite strand, we chose the *pmrR* promoter mutant, which does not express the PmrR peptide and is expected to retain an intact 3' structure in *pmrB* mRNA, rather than a $\Delta pmrR$ mutant.) Likewise, mutations in *cpxR* and/or *degP* also became severe when they were combined with the *ugd pmrR* mutant (Fig. 2B), which permits the LpxT-mediated formation of 1-PP lipid A efficiently (Kato et al., 2012). Thus, these results confirmed that the PmrA-

activated LPS modifications are the first layer of cell defense against excess ferric iron. Because the role of Cpx to *Salmonella* growth under high ferric iron conditions was not observed until the first layer of iron response was hampered, these results suggests that Cpx is one of the second layers of envelope stress response that can adapt to high Fe^{3+} .

Next, for the cell growth assay in the presence of various concentrations of ferric iron, we included a mutant of the *yajA* gene (Fig. 3) that is also under the control of the

CpxR/CpxA system in *E. coli* (Yamamoto and Ishihama, 2006) and *S. enterica* (Fig. S1). Note that the *cis* elements recognized by the CpxR protein and RNA polymerase are conserved among *E. coli*, *S. enterica*, and their closely related bacteria (Fig. S1) and that the location of the copper responsive CpxR-dependent transcription start site (TSS) at the *yqjA* promoter in *E. coli* (Yamamoto and Ishihama, 2006) matches well with one of two TSSs determined at the corresponding promoter on the *S. enterica* genome (Fig. S1) (Kim et al., 2012; Ramachandran et al., 2012). The growth rate of the quadratic mutant *ugd pmrR degP yqjA* was similar to that of the quadratic mutant in the presence of all the concentrations of Fe^{3+} *ugd pmrR degP cpxR* (Fig. 3), suggesting that, in addition to DegP, the most important contributor under the control of CpxR for the permissive growth of the *ugd pmrR* mutant (Figs. 2B and 3) was YqjA. In contrast, growth defects in the double mutants, *degP yqjA* and *degP cpxR*, were compa-

rable to that of the wild-type under those high iron conditions (Figs. 2B and 3). This also supports the hypothesis that Cpx is a second layer that can tolerate high iron toxicity after the PmrA/PmrB system layer. A mutation in the *copR* (*yedW*) gene encoding the cognate response regulator of the sensor CopS (YedV) (Wood et al., 1998; Yamamoto and Ishihama, 2005) was combined with the *ugd pmrR* double mutant and the *ugd pmrR degP* triple mutant strains to assess its involvement in the permissive iron growth phenotype (Fig. 3). However, the effect of the *copR* mutation was rather silent to those epistatic growth patterns in the presence of Fe³⁺ (Fig. 3).

Distinct epistatic patterns observed in the growth of the strains defective in PmrA- and CpxR-regulated loci in the presence of various concentrations of Fe³⁺, Cu²⁺, and Zn²⁺

Because high concentrations of Cu^{2+} or Zn^{2+} sensitize

Fig. 5. Effect of Fe^{3+} , Cu^{2+} , and Zn^{2+} on the activity of the CpxR-activated *cpxP* promoter.

The luminescence induction ratio of wild-type and the *cpxR* mutant was determined following growth for 8 h in an N-minimal medium at pH 7.7 with $10 \mu\text{M}$ Mg^{2+} in the absence or presence of various concentrations of FeCl_3 , CuSO_4 , and ZnSO_4 . The ratio shown by the y-axis corresponds to the relative luminescence levels obtained from wild-type or *cpxR* mutant cells containing pLUX1097-*p_{cpxP}* grown in the presence of various concentrations of metals, FeCl_3 , CuSO_4 , and ZnSO_4 to that from wild-type cells containing pLUX1097-*p_{cpxP}* grown in the absence of these metals. Solid and dashed lines indicate the values of wild-type cells containing pLUX1097-*p_{cpxP}*, and the *cpxR* mutant cells containing pLUX1097-*p_{cpxP}*, respectively. Data correspond to the mean of three independent experiments performed in triplicate and error bars represent standard deviations.

the *cpxRA*-null mutant in *E. coli* (Yamamoto and Ishihama, 2006), we compared the epistatic interactions that were exhibited in the growth of strains with combinations of the *pmrR*, *ugd*, *degP*, *cpxR*, *yqjA*, and *copR* mutations in the presence of various concentrations of those metals in addition to Fe^{3+} . First of all, the generally toxic metals to organisms, Cu^{2+} and Zn^{2+} , partially inhibited the growth of wild-type at concentrations of $400 \mu\text{M}$ and $100 \mu\text{M}$, respectively (Figs. 4B and 4D), although Fe^{3+} rather promoted the growth of wild-type over a wide range of concentrations ($100 \mu\text{M}$ to 3 mM ; Fig. 3). In agreement with that reported in the case of *E. coli* (Yamamoto and Ishihama, 2006), *cpxR* was crucial for *Salmonella* cell growth, also in the presence of the inhibitory concentration (i.e., $400 \mu\text{M}$) of Cu^{2+} , independently to the function of *ugd*- and *pmrR*-dependent lipid A modification (i.e., prevention of 1-PP formation), while the *ugd* and *pmrR* gene products somewhat contribute to bacterial cell growth in the mutant background of *cpxR*, but not *degP*, *yqjA* or *copR* (Fig. 4B). Thus, it is likely that the *cpxR* gene (or an unknown CpxR-regulated gene other than *degP* and *yqjA*) is predominant over the *ugd* and *pmrR* genes to permit better cell growth in the presence of Cu^{2+} (Fig. 4B). This is in contrast to the patterns observed in the presence of Fe^{3+} , where the effect of the *cpxR* mutation was not evident until the *pmrR* and/or *ugd* mutants were combined (Figs. 2 and 3). Then, the epistatic growth patterns of respective mutants in the presence of Zn^{2+} seem to be rather less reproducible and less valid than those in the presence of Fe^{3+} , and sort of inbetween those with Fe^{3+} and Cu^{2+} : mutations in *ugd*, *pmrR*, *degP*, *cpxR*, *yqjA* tend to accelerate a mutually inhibitory action of Zn^{2+} on *Salmonella* cell

growth, while only a single mutation in *cpxR* (or *yqjA*) rendered cells more sensitive to Zn^{2+} than wild-type, independently to the roles of *ugd* and *pmrR* genes (Figs. 4C and 4D). In contrast to the action of Fe^{3+} and Cu^{2+} , Zn^{2+} somewhat sensitized the strain with a single mutation in *copR* (Fig. 4D), which has been shown to be involved in the response and adaptation to reactive oxygen species (ROS), hypochlorous acid, and H_2O_2 , in *E. coli* (Gennaris et al., 2015; Urano et al., 2015). This may suggest that Zn^{2+} , but not Fe^{3+} and Cu^{2+} , produce a significant amount of ROS such as H_2O_2 , which may, in turn, solely produce cellular toxicity, including DNA damage and induced mutations, a variable for our assay, when mixed with growing bacterial cells. However, this does not necessarily mean that ROS is the only factor inhibiting cell growth of the *copR* mutant in the presence of Zn^{2+} , and that ROS does not contribute at all to the inhibitory effects of Fe^{3+} and Cu^{2+} on the cell growth of strains with multiple mutations in those genes under the controls of Pmr and CpxR. Overall, the Fe^{3+} -dependent epistasis phenomena between the PmrA-dependent loci and the CpxR-dependent loci limit the kind of metal species by definition.

CpxR-activated cpxP promoter is induced by Cu^{2+} and Zn^{2+} , but not by Fe^{3+}

Because the *cpxR* gene contributes cell growth in the presence of Cu^{2+} , Zn^{2+} , and Fe^{3+} to some extent, we hypothesized that Fe^{3+} , as well as Cu^{2+} and Zn^{2+} , induce the CpxR/CpxA system in *S. enterica*. To monitor the status of the CpxR/CpxA system, we selected the promoter of the *cpxP* gene and cloned it in front of the *luxCDABE* reporter genes in the pLUX1097 plasmid. The choice of this promoter was because it is likely to be directly regulated only by CpxR as a transcriptional regulator. The luminescence induction ratio of wild-type harboring the plasmid construct with *luxCDABE* under the control of the *cpxP* promoter, pLUX1097-*p_{cpxP}*, was 1.6×10^3 fold higher than that of the *cpxR* mutant harboring pLUX1097-*p_{cpxP}* (Fig. 5), suggesting that the CpxR/CpxA system is highly active in an N-minimal media, pH 7.7, with $10 \mu\text{M}$ MgCl_2 , even in the absence of those metals. Then, the relative luminescence levels of wild-type harboring pLUX1097-*p_{cpxP}* were induced 3.8 and 3.2 fold (when growth rates were decreased from 1 to 0.28 and 0.33, respectively) in the presence of 1 mM Cu^{2+} and 0.2 mM Zn^{2+} , respectively (Fig. 5). However, such an increase was abolished in the *cpxR* mutant, therefore indicating that the Cu^{2+} - and Zn^{2+} -mediated induction of the *cpxP* promoter is CpxR-dependent. By contrast, in the presence of 3 mM Fe^{3+} , the relative luminescence was decreased to approximately half (0.56 fold) the level of that in the absence of Fe^{3+} (Fig. 5). Even in the *cpxR* mutant, 3 mM of Fe^{3+} repressed the relative luminescence to 0.51 fold levels, which was 0.57×10^{-3} fold of levels in wild-type in the presence of 3 mM of Fe^{3+} . Namely, the repressive effect of Fe^{3+} was Cpx-independent. These results suggest that the CpxR/CpxA system is already active enough to contribute to *Salmonella* cell growth in the presence of high Fe^{3+} although it cannot be an inducing stress of the system of wild-type cells, at least in these assay conditions. We attempted to measure the effect of Fe^{3+} on the activity of the *cpxP* pro-

Fig. 6. The crucial role of the 1-PP moiety of lipid A in Fe^{3+} binding and the Fe^{3+} -dependent epistatic growth phenotype.

(A) Relative growth rate of isogenic *pmrR*⁺ and *pmrR* promoter mutants with deletions in the *ugd*, *degP*, *cpxR*, or *lpxT* genes (or a combination of these genes) following growth for 12 h in an N-minimal medium at pH 7.7 with 10 μM Mg^{2+} in the presence of 0.4 mM FeCl_3 , 0.4 mM CuSO_4 , or 0.1 mM ZnSO_4 . The bacterial growth (OD_{595}) was determined in $\Delta degP$ *ugd* mutant (AK1015), $\Delta degP$ *ugd* *pmrR* promoter mutant (AK1017), $\Delta lpxT$ $\Delta degP$ *ugd* mutant (AK1590), $\Delta lpxT$ $\Delta degP$ *ugd* *pmrR* promoter mutant (AK1591), $\Delta cpxR$ $\Delta degP$ *ugd* mutant (AK1029), and $\Delta cpxR$ $\Delta degP$ *ugd* *pmrR* promoter mutant (AK1031), $\Delta lpxT$ $\Delta cpxR$ $\Delta degP$ *ugd* mutant (AK1036), and $\Delta lpxT$ $\Delta cpxR$ $\Delta degP$ *ugd* *pmrR* promoter mutant (AK1037) strains. The growth rate was determined as described in Fig. 3. Data correspond to the mean of three independent experiments performed in triplicate and error bars represent standard deviations. (B) Thin layer chromatography (TLC) analysis of ^{32}P -labeled lipid A from wild-type (14028s) and *pmrR* promoter mutant (EG15437) *Salmonella*. Bacteria were grown in an N-minimal medium at pH 7.7 containing 10 mM Mg^{2+} and ^{32}P -orthophosphate to $\text{OD}_{600} \sim 0.4$, shifted to a medium containing 10 μM Mg^{2+} , 100 μM Fe^{3+} and ^{32}P -orthophosphate, and harvested at the designated times to isolate ^{32}P -labeled lipid A. The identity of the lipid A species is based on data in Herrera et al. (2010) and Kato et al. (2012). (C) TLC analysis of Fe^{3+} -dependent loss of the 1-PP moiety during the process of the cleavage of glycosidic linkage between KDO and in ^{32}P -labeled lipid A of LPS, which was isolated from wild-type (14028s) cells that had been grown for 10 h in an N-minimal medium, pH 7.7, with 10 mM Mg^{2+} . A non-enzymatic reaction was performed at 100°C for 30 min in the presence, or absence, of 100 μM FeCl_3 .

moter in the *ugd pmrR* mutant background also, but results were variable, possibly due to its severe growth defect (data not shown).

Predominance of the 1-PP status of the lipid A moiety of LPS on the cell surface in the Fe^{3+} -dependent growth inhibition

Lipid A is highly decorated by various combinations of the PhoP/PhoQ-dependent modifications and the PhoP/PhoQ-, PmrD-, and PmrA/PmrB-dependent modifications under low Mg^{2+} and high Fe^{3+} conditions in *S. enterica* (Chen and Groisman, 2013) (Figs. 1 and 6B, 120 min). Therefore, direct observation of the minor lipid A species that possess the 1-PP moiety in these inducing conditions is challenging, even if *cpxR*-regulated loci somewhat altered the relative quantity of 1-PP moiety per lipid A. Yet, as described above, the role of the PmrA-regulated *ugd* and *pmrR* gene products, which together inhibit the 1-PP formation, are robust and predominant over the action of the CpxR-regulated loci under a wide range of Fe^{3+} concentrations (Fig. 3) because they mask the contribution of Cpx. Especially, at 3 mM Fe^{3+} , the presence or absence of *ugd* and *pmrR* gene products is the only matter to the growth phenotype because essentially DegP, YqjA, and/or CpxR no longer support bacterial cell growth of the *ugd* and *pmrR* promoter mutant (Fig. 3D). In line with these observations, the growth defect of the quadratic mutant of *ugd pmrR degP cpxR* in the presence of 0.4 mM Fe^{3+} , but not 0.4 mM $CuSO_4$ or 0.1 mM $ZnSO_4$, was completely suppressed by mutating the *lpxT* gene (Fig. 6A). (Unlike Fe^{3+} , Cu^{2+} (and Zn^{2+}) may associate with moieties of 1-P and 1-PP of lipid A equally because mutation in the *lpxT* gene did not alter the cell growth patterns.) Therefore, the predominance of *ugd* and *pmrR* gene products was also a reflection of a predominance of the LpxT-dependent 1-PP role over the Cpx roles in the Fe^{3+} -dependent growth phenotype. In addition, current analyses, together with our previous report, demonstrate that 1-PP moiety of lipid A was not only essential for the initial binding of iron to the cell surface, as well as the reciprocal control between the LPS modification status and the iron responsive PmrA/PmrB system (Kato et al., 2012), but also for iron entry into the periplasmic space of the *S. enterica ugd pmrR* mutant and its derivatives to be evident for the growth phenotype under high iron conditions. This is because the sensor PmrB detects more Fe^{3+} at periplasm in the absence of the *ugd* and *pmrR* gene products in a *lpxT*-dependent manner (Kato et al., 2012) and because the absence of the periplasmic protease DegP and the inner membrane YqjA protein becomes significant only when the outer membrane permeability barrier is defective in hindering the formation of 1-PP moiety, which appears to bind Fe^{3+} more tightly than the moiety 1-P or 4'-P of lipid A does, as described below.

Chemical property of the 1-PP moiety of lipid A in the presence of Fe^{3+}

In time course experiments of the lipid A profiling in wild-type (Kato et al., 2012) (Fig. 6B) and the *pmrR* promoter mutant (Fig. 6B), we realized that the 1-PP moiety of the lipid A structure is liable to be rapidly converted to

the 1-OH moiety in samples obtained immediately (20 min) after switching the growth conditions from high Mg^{2+} to low Mg^{2+} and high Fe^{3+} before a variety of lipid A modifications appears (at time 120 min). We hypothesized that this rapid loss of 1-PP moiety from lipid A was an artifact of non-enzymatic reaction at 100°C for 30 min during lipid A preparation in the presence of contaminated Fe^{3+} from the media and that the 1-PP moiety of lipid A is chemically reactive to Fe^{3+} , or vice versa. To test these hypotheses, Fe^{3+} was added to an LPS sample that had been isolated from wild-type *Salmonella* cells grown under high Mg^{2+} conditions when the glycosidic linkage between KDO and lipid A was cleaved off at 100°C during the preparation of lipid A (Fig. 6C). As expected, the 1-PP moiety was almost completely converted to 1-OH moiety while the 1-P moiety still remained in the presence of 100 μM Fe^{3+} (Fig. 6C). This provides a strong evidence of a preferred interaction between the 1-PP moiety of lipid A and Fe^{3+} . In addition, it may suggest an alternative mode of Fe^{3+} entry into the periplasm in which the Fe^{3+} /pyrophosphate complex tears off slowly under an aerobic condition in the presence of a reducing power from an unknown resource at a physiological temperature. Note that chelated iron in the presence of oxygen and a reducing agent can lead to repetitive cleavage of macromolecules such as DNA (Enright et al., 1992; Molina and Anchordoquy, 2007) and that an Fe^{3+} /ADP chelate complex can cause lipid peroxidation of a mitochondrial lipid in a reducing agent-dependent manner in Eucaryotes (Takeshige et al., 1980). However, it is also true that Fe^{3+} -mediated bacterial cell killing of the *pmrA* mutant can still occur even under anaerobic conditions in a growth-dependent manner (Chamnongpol et al., 2002). Anyhow, because the LPS of the outer membrane is the first barrier to various toxic agents in Gram-negative bacteria, it makes sense that the controlling system of Fe^{3+} accessibility to the 1-PP moiety of lipid A in *S. enterica* is genetically predominant over cellular adaptive responses occurring more inside the bacterial cell envelope (i.e., the periplasm and inner membrane) to be resistant to high Fe^{3+} .

Conclusions

We have demonstrated the epistatic nature of mutant strains that are defective in bacterial signal transduction systems that respond to various stress conditions, including metals and in the genes under the control of them, in *S. enterica*. Some overlapping roles exist in genes under these different systems, while the genes under the control of the one system predominates over the genes regulated by another system, depending on which specific stress signals exist. More specifically, the CpxR/CpxA system independently contributes to *Salmonella* cell growth in the presence of a considerably toxic concentration of Cu^{2+} and Zn^{2+} (even for wild-type), exhibiting predominance over the PmrA-regulated *ugd* and *pmrR* genes. Then, the CpxR-activated *degP* and *yqjA* genes, as well as the *cpxR* gene, are contributors to *Salmonella* growth in the presence of a wide range of concentrations (100 μM to 1 mM) of Fe^{3+} , which are not toxic to wild-type cells at all, only when the outer membrane permeability barrier is defective in the

Ugd-mediated L-Ara4N modification of lipid A and the action of PmrR peptide, which together inhibit the LpxT-dependent generation of 1-PP lipid A (Kato et al., 2012). This epistatic phenomenon in *S. enterica* is absolutely dependent on the LpxT-catalyzed 1-PP moiety formation of lipid A as well as Fe³⁺, but not on Cu²⁺ and Zn²⁺. Interestingly, it has been reported that limited ferrous iron conditions induce the CpxR/CpxA system and that high concentrations of other metal ions, such as Cu²⁺ and Mg²⁺, which may potentially compete with cations like Fe²⁺ and Fe³⁺ on anionic surfaces, also induce transcription of the CpxR-activated *cpxP* gene in *Vibrio cholera* (Acosta et al., 2015) where orthologs of the PmrA/PmrB system and the PmrA-activated LPS modification enzymes are absent. It is also interesting to wonder whether *V. cholera* lost the *pmrAB* genes and the PmrA-regulated LPS modification genes, such as *ugd*, *pbgPE*, *pmrC*, and *pmrR*, or whether a common ancestral bacterium acquired them becoming *S. enterica* during the long-term evolution of Gram-negative bacteria. *S. enterica* may have adapted to a high Fe³⁺ environment better. In conclusion, Cpx is one of the second layers of the envelope stress response that can adapt to high Fe³⁺ conditions in *S. enterica*.

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Supplementary Materials

Supplementary figure is available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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