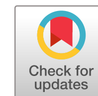
AMERICAN SOCIETY FOR MICROBIOLOGY Journal of Bacteriology®

RESEARCH ARTICLE



pqiABC and *yebST*, Putative *mce* Operons of *Escherichia coli*, Encode Transport Pathways and Contribute to Membrane Integrity

Takayuki Nakayama, Qiu-Mei Zhang-Akiyama

Laboratory of Stress Response Biology, Department of Biological Sciences, Graduate School of Science, Kyoto University, Kyoto, Japan

ABSTRACT The membranes of single-cell organisms are crucial as the first line of defense. The outer membrane of Gram-negative bacteria is an asymmetric bilayer in which lipopolysaccharides (LPSs) and phospholipids are localized in the outer and inner leaflet, respectively. This asymmetry is important for membrane integrity. In *Escherichia coli*, the Mla transport pathway maintains this asymmetry by removing phospholipids from the outer leaflet. The MlaD component of this system is a mammalian cell entry (MCE) domain protein, and *E. coli* has two other MCE domain proteins of unknown function (PqiB and YebT). Here, we show that these two proteins are components of novel transport pathways that contribute to membrane integrity. The *pqiAB* operon is regulated by SoxS and RpoS. The *yebST* operon contains *pqiAB* homologues. Here, we found a third member of the *pqi* operon, *ymbA* (*pqiC*). A PqiB-PqiC complex bridges the inner and the outer membrane, and in other bacteria, *pqiBC* genes are located in operons together with transporter proteins. We show here that simultaneous deletion of *pqiABC* and *yebST* operons in an Δ*mla* background rendered cells more sensitive to SDS-EDTA, and the SDS-EDTA sensitivity of *mla* mutants was rescued by additional copies of *pqiABC*. We also found that the *yebST* operon was induced by a defect in LPS molecules. In conclusion, PqiABC and YebST are novel transport pathways related to the Mla transport pathway and important for membrane integrity.

IMPORTANCE Membranes of bacteria are crucial for stress resistance. The composition of the *E. coli* outer membrane is asymmetric, with asymmetry maintained by the Mla ABC transport pathway. We propose that the stress-inducible *pqiABC* operon and homologous *yebST* operon, both of previously unknown function, encode transport pathway proteins related to the Mla transport pathway. Deletion of these operons rendered cells more sensitive to membrane stress, and additional copies of *pqiABC* suppressed the SDS-EDTA sensitivity of *mla* mutant strains. We found that *yebS′*-*′lacZ* fusion was activated in mutant strains with defective LPS molecules.

KEYWORDS SoxS, membranes, oxidative stress, *pqiABC*, *yebST*

Received 9 August 2016 **Accepted** 4 October 2016

Accepted manuscript posted online 17 October 2016

Citation Nakayama T, Zhang-Akiyama Q-M. 2017. *pqiABC* and *yebST*, putative *mce* operons of *Escherichia coli*, encode transport pathways and contribute to membrane integrity. J Bacteriol 199:e00606-16. https://doi.org/10.1128/JB.00606-16.

Editor R. L. Gourse, University of Wisconsin—Madison

Copyright © 2016 American Society for Microbiology. All Rights Reserved.

Address correspondence to Qiu-Mei Zhang-Akiyama, qmzhang@kingyo.zool.kyoto-u.ac.jp.

Escherichia coli, a Gram-negative bacterium, has an inner membrane (IM) and an outer membrane (OM). Since these membranes separate essential cellular components from the environment, they are important for stress resistance (1, 2). The OM of *E. coli* is an asymmetric bilayer in which lipopolysaccharides (LPSs) and phospholipids (PLs) are localized in the outer and the inner leaflet, respectively (3). Lipophilic molecules easily penetrate a bilayer of PLs. Because of the low fluidity of lipid A (the lipid portion of LPSs), the asymmetric OM performs a barrier function against lipophilic molecules (3). Mutant strains with defective LPSs are also sensitive to hydrophilic molecules, presumably due to transient cracks in the OM (3). Therefore, disruption of

the asymmetric composition reduces the effectiveness of the barrier and renders cells sensitive to various stressful conditions (4). In unstressed cells, the Mla ABC transport pathway maintains the OM asymmetry by removing PLs from the outer leaflet. Under stressed conditions, two other factors, PldA phospholipase and PagP palmitoyl transferase, also remove PLs from the OM (4).

MlaD, a periplasmic component of the Mla pathway (4), has a mammalian cell entry (MCE) domain. *mce* genes were originally found in *Mycobacterium tuberculosis*, and one of them (*mce1*) enables a nonpathogenic *E. coli* strain to invade macrophages and HeLa cells (5). Other proteins with MCE domains are found in several transport pathways (MCE proteins are listed at http://www.ebi.ac.uk/interpro/entry/IPR003399). The actual function(s) of these domains is not known, but it was shown that InvX (a 72-amino-acid fragment containing the MCE domain of Mce1) is sufficient for extensive association with HeLa cells (6). *M. tuberculosis* has four *mce* operons, and mutations in these operons attenuate the virulence of *M. tuberculosis* (7).

According to the InterPro Database, *E. coli* has two other MCE proteins (PqiB and YebT). The *pqiB* gene is a part of the *pqiAB* (paraquat-inducible) operon, which is induced by paraquat in a SoxS-dependent manner (8, 9). Paraquat (methyl viologen [MV]) is an agricultural chemical that generates superoxide radical (10). Starvation also induces the *pqiAB* operon via RpoS (8). The *yebT* gene is a homologue of *pqiB*, but they are quite different in length (2,634 bp and 1,641 bp, respectively). *yebT* forms an operon with the *yebS* gene, which is highly homologous to *pqiA*. No functions of these genes or phenotypes of mutant strains have been reported yet. Because the *pqiAB* operon is conserved in many pathogenic bacteria (including *Vibrio cholera*, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa*) (11) and *mce* operons encode virulence factors in *M. tuberculosis*, it is important to identify their functions.

In this study, we obtained several pieces of evidence showing that the *pqiABC* and *yebST* operons encode transport pathway proteins that are functionally related to those in the Mla pathway. The localization and structure of these components are suitable for functioning in transport pathways, and the genomic organization of their homologous genes also supports our hypothesis that that these two operons encode transport pathway proteins functionally related to those in the Mla pathway. Deletion of these operons reduced membrane integrity, and additional copies of *pqi* genes rescued *mla* mutant strains.

RESULTS

***ymbA* (*pqiC*), a DUF330 family lipoprotein gene, is a member of the *pqiAB* operon.** The *pqiAB* operon consists of two membrane protein coding genes, which are induced by SoxS under oxidative conditions (8). Examination of the DNA sequence showed that the stop codon of *pqiB* overlaps with the start codon of the downstream gene (*ymbA*) (Fig. 1A). Therefore, it was predicted that *pqiB* and *ymbA* are translationally coupled and form an operon (12). To test this possibility, we constructed reporter plasmids and examined their MV responsiveness. YmbA has a putative lipobox, an export signal peptide for OM lipoprotein (13). We fused the *ymbA* gene with *phoA*, an *E. coli* alkaline phosphatase (AP) gene. PhoA becomes enzymatically active only when exported to the periplasm where the required intrachain disulfide bond can be formed (14). The resultant pPAF-*pqiAB-ymbA* plasmid contains the sequence from 420 bp upstream of the *pqiA* gene to the last 5 bp of the *ymbA* gene (Fig. 1A). AP assays were carried out as described in reference 14. High AP activities were detected from cells expressing this fusion protein, indicating that the putative lipobox acts as an export signal. Furthermore, the expression of the YmbA-PhoA fusion protein was induced by MV, and this response was eliminated by *soxS* gene disruption (Fig. 1B). In addition, the response to MV was also eliminated by deletion of the SoxS-binding site in the *pqiA* promoter region (−420 to −305, upstream of the BglII site) (Fig. 1) (9). These results indicate that *ymbA* is a member of the *pqiAB* operon. In this study, we hereafter call this gene *pqiC*.

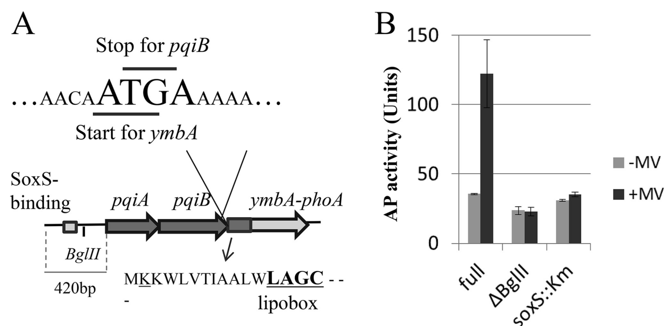


FIG 1 Analysis of *ymbA* (*pqiC*)′-′*phoA* reporter fusions. (A) A schematic diagram of the reporter construct. The *ymbA* gene was fused in frame with the *phoA* gene without its export signal. The upstream 420 bp of the *pqiA* gene was also included. In the region upstream of the BglII site, there is a SoxS-binding site. The N-terminal amino acid sequence of PqiC is shown; the putative lipobox is represented in bold. (B) Assays for the translational *ymbA*′-′*phoA* fusions. Exponentially growing *E. coli* cells harboring the *ymbA*′-′*phoA* fusion were treated with 50 μM MV for 1 h. The cells were then washed once, and whole-cell extracts were prepared and used for AP activity measurement. Full and *soxS*::Km^r, BW25113 and JW4023 (*soxS*::Km^r) harboring the reporter described in panel A; BglII, BW25113 harboring a reporter lacking the SoxS-binding site in the *pqiA* promoter.

***pqiB* and *pqiC* homologues comprise operons with transporter protein genes.** By analyzing homologous genes in other bacteria, we found that in *Legionella pneumophila*, a human-pathogenic bacterium, homologues of the ABC transporter genes *mlaFE* constitute an operon with the homologues of *pqiB* and *pqiC* genes (Fig. 2) (these data are available in the GenBank database [https://www.ncbi.nlm.nih.gov/gene/19833613]). This fact suggests that PqiB and PqiC may deliver a substrate(s) to a transporter protein. Indeed, like MlaD protein, a substrate-binding protein of the Mla pathway, PqiB protein also has MCE domains. MCE domains were found in substrate-binding proteins of lipid transporter pathways (http://www.ebi.ac.uk/interpro/entry/IPR003399). However, the amino acid sequence of PqiA has no similarity with that of known transporter proteins.

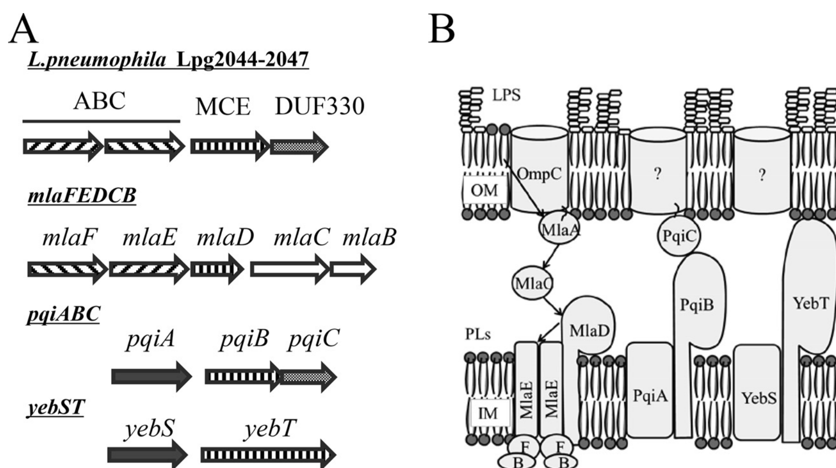


FIG 2 Schematic diagrams of *pqiABC* and related loci. (A) Genomic context. Homologous genes are shown using the same graphic pattern. In *E. coli mlaFEDCB*, *pqiABC*, and *yebST* operons, there is one MCE protein gene in each operon (arrow with vertical lines). MlaF and MlaE are components of an ABC transporter, and MlaD and MlaC deliver transported substrates. In *L. pneumophila*, *pqiBC* homologues are located downstream of *mlaFE*-like genes. Unlike in *E. coli*, a permease component (*mlaE*) is located in front of an ABC-binding component (*mlaF*). (B) Localization and functions of product proteins. Mislocalized PLs are removed by the OmpC-MlaA complex and delivered by other components as represented by arrows. Because OmpC and MlaA are expressed from other loci, they are not included in panel A. Predicted localizations of PqiABC and YebST are also shown in this figure. Details are discussed in later sections.

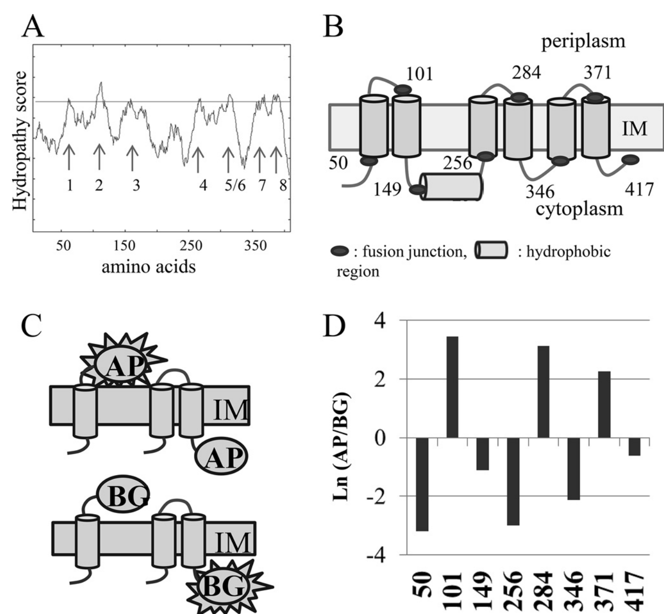


FIG 3 Topological analysis of PqiA protein. (A) Kyte-Doolittle hydropathy plot. The window number is 19. Predicted TM segments are indicated by arrows. Because there is no hydrophilic region between segments 5 and 6, this region is represented as a single TM segment. (B) Topological model predicted from the positive inside rule and the hydropathy plot. There is clustering of basic residues in hydrophilic regions 1, 3, 4, 6, and 8, which suggests cytoplasmic localization of these segments. The fusion points and hydrophobic segments are indicated by gray ovals and cylinders, respectively. (C) Schematic diagram of the PhoA/LacZ fusion system. Both AP and LacZ fusions were constructed for each fusion point. AP is active when it is localized in the periplasm, and BG is active when it is localized in the cytoplasm. (D) Relative activities of *pqiA′-′lacZ* and *pqiA′-′phoA* fusions. These fusion genes were placed under the control of an MV-inducible *pqiA* promoter. Exponentially growing *E. coli* cells harboring *pqiA′-′lacZ* and *pqiA′-′phoA* fusion-expressing vectors were treated with 50 μM MV for 1 h. Relative activities were calculated as described in Materials and Methods. Positive and negative values indicate periplasmic and cytoplasmic localization, respectively. Experiments were conducted at least three times, and representative data are shown.

PqiA is an inner membrane protein with six transmembrane segments. As mentioned above, we did not detect sequence homology between PqiA and known transporter proteins. Therefore, we investigated whether PqiA has any structural similarity with transporter proteins. In the BioCyc Database (http://biocyc.org/), PqiA was described as a protein with eight transmembrane (TM) segments, each with a length of about 20 amino acids. We also detected corresponding hydrophobic segments using several hydropathy analyses (Fig. 3A). However, there is no hydrophilic region between TM segments 5 and 6. Therefore, we considered it likely that these segments together comprise a single long TM segment.

It is known that in integral membrane proteins, positively charged amino acids (Lys and Arg) are abundant in the cytoplasmic portion, compared with the periplasmic portion (15). Based on this “positive inside rule,” the clustering of basic residues in hydrophilic regions 1, 3, 4, 6, and 8 suggests that these segments are localized in the cytoplasm (Fig. 3B). Most ABC transporters are known to comprise six TM segments (16).

To test our hypothesis that hydrophilic regions 1, 3, 4, 6, and 8 are localized in the cytoplasm, we constructed fusion protein-expressing vectors in which the end of the C terminus of each hydrophilic region was fused to β-galactosidase (BG [LacZ]) or alkaline phosphatase (AP [PhoA]) without its signal sequence. In contrast to AP, BG is enzymatically active only in the cytoplasm. Therefore, by comparing the activities of the two proteins produced by fusion with that of the test peptide at the same point, we can determine the tested region’s localization (Fig. 3C). The results revealed higher AP activities at three points (101, 284, 371) and higher BG activities at the other five points. This is consistent with the six-transmembrane model. As predicted, hydrophobic

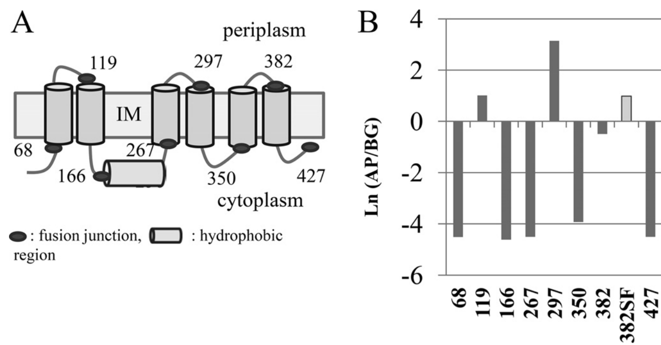


FIG 4 Topological analysis of YebS protein. (A) Topological model predicted from the positive inside rule. The fusion points and hydrophobic segments are indicated by gray ovals and cylinders, respectively. (B) Relative activities of *yebS′-′phoA′-lacZα* fusions. These fusion genes and the *ϕ80′lacZ*ΔM15 allele (LacZω fragment) were both IPTG inducible. Exponentially growing *E. coli* cells harboring *yebS′-′phoA′-lacZα* fusion-expressing vectors were treated with 0.25 mM IPTG for 1 h. Relative activities were calculated as described in Materials and Methods. Positive and negative values indicate periplasmic and cytoplasmic localization, respectively. 382SF, PhoA-LacZα sandwich fusion at 382Q. Experiments were conducted at least twice, and representative data are shown.

segment 3 does not cross the IM, and the hydrophobic segment 5/6 is a single long TM segment (Fig. 3B and D).

Structural similarity between PqiA and YebS. Based on the positive inside rule, the membrane topology of YebS protein was predicted to be similar to that of PqiA protein (Fig. 4A). To test our hypothesis that YebS and PqiA are both six-transmembrane proteins with similar topology, we constructed YebS-PhoA-LacZα dual reporters (17). In this construct, the N-terminal segment of LacZ (LacZα fragment) was fused to the C-terminal segment of PhoA. Only when the fusion segment is cytoplasmic is it accessible to the C-terminal segment of LacZ (LacZω fragment) expressed from the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *ϕ80′lacZ*ΔM15 allele of the host strain. The junction points were chosen based on the alignment of the PqiA and YebS amino acid sequences. Because there were no known conditions for inducing the *yebS* promoter at the time this experiment was performed, these fusions were placed under the control of the *lac* promoter.

Other than the fusion at 382Q (corresponding to 371M of PqiA), most fusions exhibited activities similar to those of PqiA fusions (Fig. 4B). However, the fusion at 382Q showed high-level BG and medium-level AP activities (Fig. 4B). This result is not consistent with the predicted model or the periplasmic localization of PqiA371M. There are some examples in which the C-terminal segment is known to be necessary for proper localization of the N-terminal segment (18). Therefore, instead of the C-terminally truncated fragment, we inserted the PhoA-LacZα fragment between 382Q and 383I of YebS (producing a sandwich fusion). This fusion protein exhibited much lower BG activity than that of the C-terminally truncated fusion (Fig. 4B). This result suggests that the periplasmic localization of 382Q was unstable without the C-terminal segment. Collectively, these results indicate that YebS and PqiA are both six-transmembrane proteins with similar topology.

Large portion of PqiB was localized in the periplasm. If PqiB and PqiC proteins deliver substrates to a transporter, they should be localized in the periplasmic space. PqiB was predicted to be inner membrane localization, with a single TM segment (Fig. 5A). To determine the localization of PqiB, a C-terminal PhoA-LacZα dual fusion was constructed (17). As shown in Fig. 5B, the PqiB-PhoA-LacZα fusion exhibited high AP and low BG activities, indicating that a large portion of PqiB, including the MCE domains, is exported to the periplasm (Fig. 5E).

yebT is a homologue of *pqiB*, and its product also has a single TM segment and multiple MCE domains (Fig. 5A). We constructed a YebT-PhoA-LacZα fusion and confirmed its periplasmic localization (Fig. 5B).

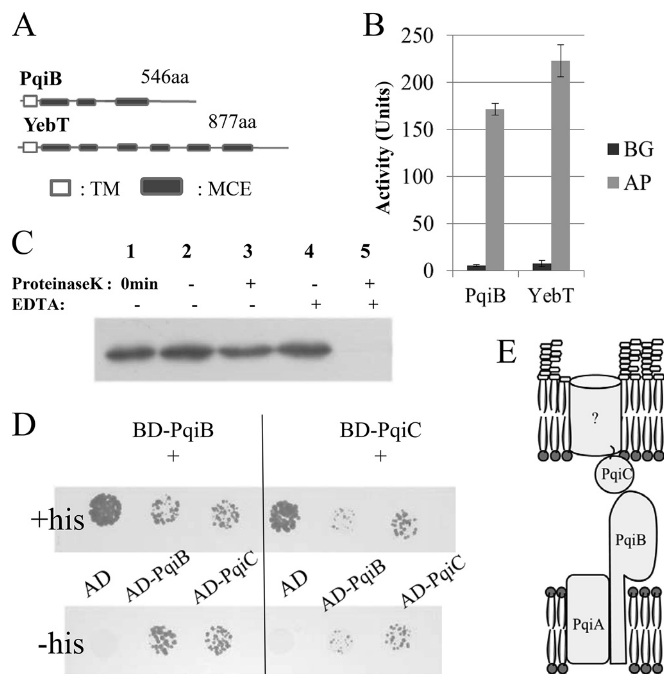


FIG 5 Analysis of localization and interactions of PqiB, YebT, and PqiC proteins. (A) Domain composition of PqiB and YebT proteins. TM, transmembrane domains; MCE, MCE domains; aa, amino acids. (B) Absolute activities of *pqiB′-′phoA′-lacZα* and *yebT′-′phoA′-lacZα* fusions. These fusion genes and the ϕ80′*lacZ*ΔM15 allele (LacZω fragment) were all IPTG inducible. Exponentially growing *E. coli* cells harboring fusions were treated with 0.25 mM IPTG for 1 h. (C) Protease accessibility assay of PqiC protein. C-terminally HA-tagged PqiC protein (PqiC-HA) was expressed from the pTrc99A vector. Exponentially growing *E. coli* cells were treated with 1 mM IPTG for 1 h and then washed and resuspended with Tris-sucrose buffer (pH 8.0). Protease treatments were conducted at 25°C for 30 min. Lanes: 1, 0 min; 2, distilled water; 3, 50 μg/ml proteinase K; 4, 5 mM EDTA; 5, 5 mM EDTA plus 50 μg/ml proteinase K. (D) Yeast two-hybrid assay. The host yeast strain HF7c is a histidine auxotroph, and interaction between AD and BD fusions leads to expression of the histidine synthesis gene. Transformants were spotted on media with or without histidine (+his and −his, respectively). (E) Predicted localization of PqiABC proteins. Experiments were conducted at least twice, and representative data are shown.

PqiC is a periplasmic protein anchored with the OM. PqiC protein has a signal peptide known as a lipobox (Fig. 1A). At first, lipoprotein precursors are transported to the periplasmic side of the IM. If the second residue is Asp, the lipoprotein remains in the IM. Otherwise, the lipoprotein is transported to the OM (13). Since the second residue is not Asp (Fig. 1A), PqiC is predicted to be localized in the outer membrane. Although all known lipoproteins of *E. coli* K-12 face the periplasm, surface-exposed lipoproteins have been discovered in some other Gram-negative bacteria (19). Therefore, we conducted a protease accessibility assay to test its periplasmic localization. *E. coli* cells expressing PqiC protein with a C-terminal hemagglutinin (HA) tag were treated with proteinase K in Tris-sucrose buffer. Whole-cell extracts were analyzed by Western blotting. When cells were intact, PqiC-HA protein was not digested by proteinase K (Fig. 5C, lanes 2 and 3). It is known that EDTA causes permeabilization of the OM, thus allowing access of proteinases to the periplasm (20). In the presence of 5 mM EDTA, PqiC-HA protein was digested by proteinase K (Fig. 5C, lanes 4 and 5). Thus, PqiC-HA protein was not proteinase K resistant or aggregated in the cytoplasm but rather was localized in the periplasm and inaccessible to proteinase K in intact cells (Fig. 5E).

PqiB and PqiC bridges the inner and outer membranes. Considering their localizations, PqiB and PqiC might interact to bridge the IM and the OM (Fig. 5E). To test this hypothesis, we conducted a yeast two-hybrid assay to investigate whether PqiB and PqiC interact. The host yeast strain HF7c is a histidine auxotroph, and interaction between AD and BD fusions leads to expression of a histidine synthesis gene (HIS3). When histidine was present in the medium, due to the resultant toxicity, GAL4AD-PqiB-

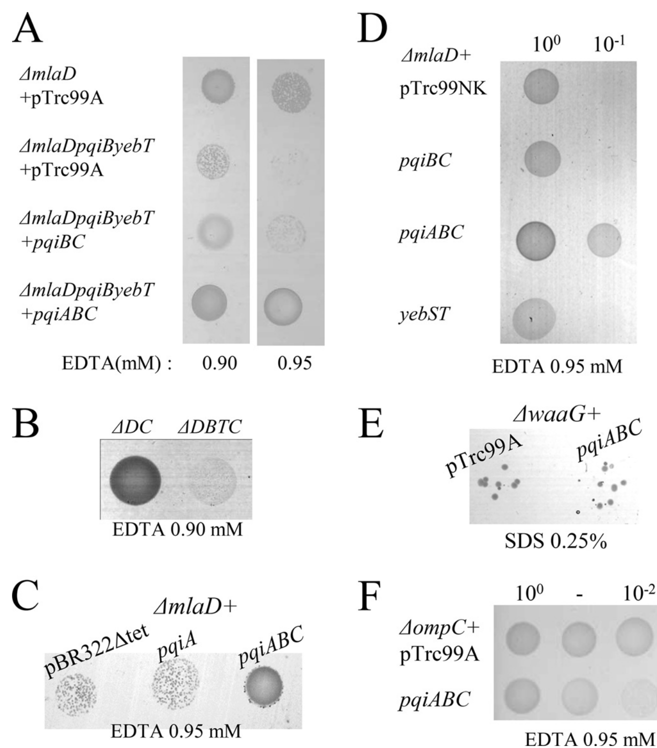


FIG 6 Assays for SDS-EDTA sensitivity. Unless otherwise stated, *E. coli* cultures in stationary phase were spotted onto LB plates containing 0.1% SDS and EDTA. The plates were then incubated at 37°C for 16 h. Expression from plasmid vectors was not induced by additional agents. (A) Δ*mlaD* and Δ*mlaD pqiB yebT* strains. These cells harbored pTrc99A-derived vectors. Undiluted cultures were spotted. (B) Δ*mlaD ompC* (ΔDC) and Δ*mlaD pqiB yebT ompC* (ΔDBTC) strains. Undiluted cultures were spotted. (C) PqiA- or PqiABC-expressing cells. Δ*mlaD* cells harbored pBR322-derived vectors. *pqiA* or *pqiABC* genes were under the control of the native *pqiA* promoter. Tenfold-diluted cultures were spotted. (D) PqiBC-, PqiABC- or YebST-expressing cells. Δ*mlaD* cells harbored pTrc99A-derived vectors. (E) Δ*waaG* cells harbored pTrc99A-derived vectors. Undiluted cultures were spotted onto LB plates containing 0.25% SDS. (F) Δ*ompC* cells harbored pTrc99A-derived vectors. Experiments were conducted at least twice, and representative data are shown.

or GAL4AD-PqiC-expressing cells grew poorly compared with GAL4AD-expressing cells (Fig. 5D). However, the growth of fusion protein-expressing cells was not affected by withdrawal of histidine from the medium. These results indicate that PqiB and PqiC proteins form not only a hetero-oligomer(s) but also homo-oligomers. This bridge formation is consistent with the substrate delivery hypothesis.

PqiABC and YebST are important for membrane integrity. Although the *pqiABC* operon is induced by oxidative stress and under starvation conditions, mutant strains are not sensitive to these stresses (8). *E. coli* has a homologous *yebST* operon; however, even simultaneous deletion of the *pqiABC* and *yebST* operons did not affect its sensitivity to these stresses (data not shown).

As discussed above, in *Legionella pneumophila*, *mlaFE* genes constitute an operon together with *pqiBC* genes. Therefore, we predicted that *pqiABC* is functionally related to the Mla transport pathway. In the presence of EDTA, the OM is destabilized due to repulsion of negatively charged LPS molecules. In such a condition, misplaced PLs are deleterious, and mutant strains of *mla* genes exhibit severe SDS-EDTA sensitivity (4).

Although sole disruption of the Mla pathway renders cells hypersensitive to SDS-EDTA, we found that only simultaneous deletion of both the *pqiABC* and *yebST* operons in the Δ*mla* background affected SDS-EDTA sensitivity (Fig. 6A). This hypersensitivity was complemented by the *pqiBC*-expressing plasmid (Fig. 6A). However, due to its toxicity, the *yebT*-expressing plasmid did not complement the hypersensitivity (data not shown).

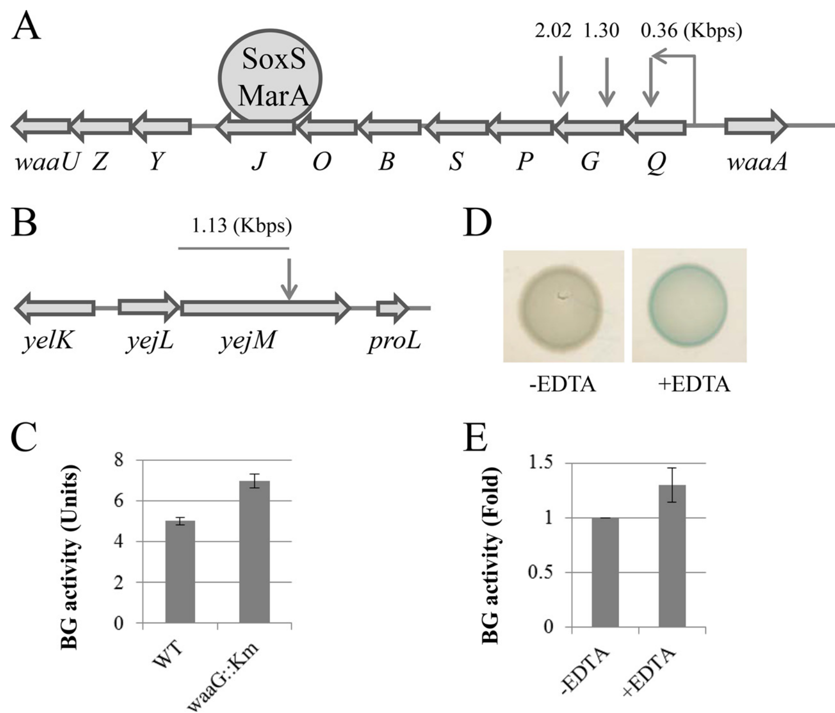


FIG 7 Screening for *yebST*-inducing conditions. *E. coli* cells harboring pMC1403-*yebS* were infected with a mini-Tn*10*-containing λ phage (λ1098). Blue colonies were isolated, and λ1098 insertion sites were determined. (A) Approximate positions of mini-Tn*10*-inserted sites in the *waa* operon (counted from the start codon of *waaQ*). Because of the polar effect, the expression of downstream genes was also affected. The downstream part of this operon has a SoxS/MarA-inducible promoter. (B) Approximate positions of mini-Tn*10*-inserted sites in the *yejM* gene (counted from the start codon of the *yejM gene*). (C) Stationary-phase cultures of *E. coli* cells harboring pMC1403-*yebS* were diluted 100-fold with flesh medium and incubated at 37°C for 7 h. Experiments were conducted twice. (D) BW25113 cells harboring pMC1403-*yebS* were grown to stationary phase. Then 4 μl of this culture was spotted onto LB–Amp–X-Gal plates with or without 1 mM EDTA. The plates were then incubated at 37°C for 16 h. (E) BW25113 cells harboring pMC1403-*yebS* were treated with 1 mM EDTA for 75 min. Experiments were conducted twice.

It was reported that the Mla pathway depends on OmpC porin for its function (21). However, even in the Δ*mlaD ompC* background, deletion of both the *pqiABC* and *yebST* operons rendered cells sensitive to SDS-EDTA (Fig. 6B).

Next, we performed several complementation experiments. In these experiments, expression from plasmid vectors was not induced by additional agents, but genomic copies of these genes were intact. Additional copies of *pqiABC* but not of *yebST* partially rescued the Δ*mlaD* mutant strain (Fig. 6C and D). However, the *pqiABC*-expressing vector did not rescue the SDS sensitivity of the Δ*waaG* strain (Fig. 6E). Moreover, the *pqiABC*-expressing vector rendered the Δ*ompC* strain more sensitive to SDS-EDTA (Fig. 6F). Additional copies of PqiA alone or PqiBC did not rescue the Δ*mlaD* mutant strain (Fig. 6C and D).

***yebST* operon was induced by a defect in lipopolysaccharide molecules.** Consistent with the toxicity of YebT, expression of *yebS*′-′*lacZ* fusion was very weak under the nonstressed condition (Fig. 7C). However, the fact that deletion of the *yebT* gene affected SDS-EDTA sensitivity indicated that the *yebST* genes are not pseudogenes. To search for a factor(s) which controls the expression of the *yebST* operon, we constructed a mini-Tn*10*::Tet^r insertion library. BW25113 cells harboring pMC1403-*yebS* were infected with λ1098 (22) and incubated on Luria-Bertani (LB)–ampicillin (Amp)–tetracycline (Tet)–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates. We thereby obtained several blue colonies, and among them we found three independent insertions in the *waa* operon (Fig. 7A). It has been reported that *waaG* and *waaP* mutants have truncated LPS molecules and are highly sensitive to SDS (23). We measured BG activity of a *waaG* deletion mutant and confirmed that expression of

yebS′-′lacZ fusion was higher than that in the wild-type strain (Fig. 7C). We also found a C-terminal deletion of the *yejM* gene, which causes a defect in lipid A (a lipid component of LPSs) synthesis (Fig. 7B) (24). LPS molecules are stabilized by bivalent cations, and we found that *yebS′-′lacZ* fusion was also induced by EDTA (Fig. 7D and E). Most of the other blue colonies were sensitive to SDS, indicating that these clones also had defects in their membranes. *pqiA′-′lacZ* fusion was not induced by membrane stress conditions (data not shown).

DISCUSSION

SoxS is a transcriptional regulator of the AraC/XylS family and induces the expression of a large number of regulon genes that play critical roles in protecting cells from oxidative stress (25). SoxS-activated genes include those for a superoxide scavenging enzyme (*sodA*), a DNA repair enzyme (*nfo*), a pyruvate:flavodoxin oxidoreductase (*ydbK*), a transcriptional repressor of iron uptake (*fur*), oxidative stress-resistant isozymes of the tricarboxylic acid (TCA) cycle (*fumC* and *acnA*), and an efflux pump (*acrAB*) (26–28).

pqiAB were discovered as SoxS-regulated genes about 2 decades ago, but the function(s) of these genes is still unknown (8). In this study, we obtained several pieces of evidence showing that the *pqiABC* operon and its homologous *yebST* operon are transporter pathways related to the *mla* operon. The six-transmembrane topology of PqiA (Fig. 3B) and the bridge formation of the PqiB-PqiC complex (Fig. 2B and 5E) are consistent with this hypothesis.

Additional copies of PqiACB complemented the Δ*mlaD* strain, but additional copies of PqiA alone or PqiB-PqiC did not (Fig. 6C and D). This result indicates that PqiA and PqiB-PqiC work together. Because PqiB and PqiC constitute an operon together with transporter proteins in *L. pneumophila*, we consider it likely that PqiA is a permease component. While sole deletion of the Mla pathway renders cells sensitive to SDS-EDTA, only simultaneous deletion of the *pqiABC* and *yebST* operons in a Δ*mla* background affected SDS-EDTA sensitivity (Fig. 6A). This result indicates that these two pathways transport PLs ineffectively or transport different substances that have minor effects on membrane integrity.

Unlike overexpression of *pqiB*, overexpression of *yebT* was highly toxic, and additional copies of *yebST* did not suppress the SDS-EDTA sensitivity of the Δ*mlaD* strain. The most obvious difference is that there is no *pqiC* homologue (DUF330) in the *yebST* operon. Instead, the *yebT* gene is much larger than the *pqiB* gene (2,634 bp and 1,641 bp, respectively). Interestingly, *Bradyrhizobium elkanii* USDA 76 possesses a protein with both DUF330 and MCE domains (BRAEL_RS0100830). (These data are available in the GenBank database [https://www.ncbi.nlm.nih.gov/gene/23084765].) YebT may be derived from such a fusion protein and be able to bridge the two membranes by itself. Considering the fact that the Mla pathway delivers PLs and modifies membrane composition, YebT may also modify membrane composition and thus kill cells when overexpressed. Interestingly, Sutterlin et al. reported the existence of a toxic variant of the *mlaA* gene (*mlaA**) (29). Those authors considered that this toxicity was caused by traffic of lipids from the IM to the OM (the reverse orientation compared with wild-type MlaA). Such retrograde traffic deprives the IM of phospholipids. It is possible that the toxicity of overexpressed YebT is also due to such a retrograde traffic. However, conditions that rescued the *mlaA** mutant (deletion of the *lpp* gene and supplementation of Mg^{2+}) did not suppress the toxicity of YebT (data not shown). These results indicate that the causes of toxicity are different or overexpression of YebT is too toxic to be rescued by these conditions.

Our findings that both deletion and overexpression of *yebT* made cells SDS-EDTA sensitive indicated that it is important to produce the proper amount of YebST. Expression of a *yebS′-′lacZ* fusion was very weak under the nonstressed condition; however, we found that the *yebS′-′lacZ* fusion was activated by several conditions that affect LPS molecules (Fig. 7). Therefore, the presence of a moderate amount of YebST seems to be beneficial for cells, especially when there are defects in LPS molecules.

Because even simultaneous deletion of these three pathways did not affect the

TABLE 1 Bacterial and yeast strains

Strain	Genotype	Reference or source
DH5αT1R	F⁻ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1 tonA*	Invitrogen
BW25113	F⁻ Δ(*araD-araB*)*567 lacZ4787*(del)::*rrnB-3* λ⁻ *rph-1* Δ(*rhaD-rhaB*)*568 hsdR5*	39
JW4023	BW25113 *soxS*::Km^r	31
JW0934	BW25113 *pqiB*::Km^r	31
JW18222	BW25113 *yebS*::Km^r	31
JW18223	BW25113 *yebT*::Km^r	31
JW3160	BW25113 *mlaD*::Km^r	31
JW2203	BW25113 *ompC*::Km^r	31
BW25113 *pqiB yebT*	BW25113 *pqiB*::Km^r *yebT*::FRT	This study
BW25113 *mlaD pqiB*	BW25113 *mlaD*::Km^r *pqiB*::FRT	This study
BW25113 *mlaD yebT*	BW25113 *mlaD*::Km^r *yebT*::FRT	This study
BW25113 *mlaD pqiB yebT*	BW25113 *mlaD*::Km^r *pqiB*::FRT *yebT*::FRT	This study
BW25113 *mlaD ompC*	BW25113 *mlaD*::Km^r *ompC*::FRT	This study
BW25113 *mlaD pqiB yebT ompC*	BW25113 *mlaD*::FRT *pqiB*::FRT *yebT*::FRT *ompC*::Km^r	
HF7c (yeast)	*MAT***a** *ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS*::*GAL-HIS3 URA3*::(*GAL417*mers)*3-CYC1-lacZ*	BD Clontech

resistance to oxidative stress conditions, the significance of SoxS-dependent expression of the *pqiABC* operon is not clear. SoxS also regulates LPS modification genes (*waaYZ*) (30), indicating the importance of the OM for oxidative stress resistance. It is possible that the growth media used in this study do not contain a transported substrate(s). The fact that the *mce* operons of *M. tuberculosis* are virulence factors suggests that it is possible that PqiABC and YebST are necessary for transporting metabolites of the host animals (7).

MATERIALS AND METHODS

Bacterial and yeast strains and plasmids. Unless otherwise stated, *E. coli* cells were grown in Luria-Bertani (LB) medium at 37°C with vigorous shaking. When necessary, ampicillin (Amp) (100 μg/ml), kanamycin (Km) (30 μg/ml), tetracycline (Tet) (10 μg/ml), and/or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml) was added. *Saccharomyces cerevisiae* cells were grown in SD medium (1.7 g/liter yeast nitrogen base, 5 g/liter ammonium sulfate, 20 g/liter glucose, 100 mg/liter adenine, 76 mg/liter uracil, 76 mg/liter L-lysine) or YPDA medium (10 g/liter yeast extract, 20 g/liter tryptone, 20 g/liter glucose, 100 mg/liter adenine) at 30°C with vigorous shaking. When necessary, L-histidine (76 μg/ml), L-leucine (400 μg/ml), and/or L-tryptophan (400 μg/ml) was added. Bacterial strain BW25113 and its derivatives were supplied by the National BioResource Project (NBRP) (31). Double and triple mutant strains were constructed by the standard P1 transduction procedure. pCP20, a site-specific recombinase-expressing plasmid, was used to remove the kanamycin resistance gene from recipient strains (32). Other *E. coli* K-12 strains and plasmids used in this study are listed in Tables 1 and 2.

Construction of reporter vectors. The DNA fragment coding for alkaline phosphatase (nucleotide 63 to the stop codon of the *phoA* gene [33]) was amplified from the chromosome of *E. coli* BW25113 using primers that introduced SalI and HindIII restriction sites at the ends of the product. The forward primer also had recognition sites for XbaI, XhoI, KpnI, PstI, and BamHI. The *phoA* gene fragment was ligated into pKK223-3 digested with SalI and HindIII. The resulting plasmid was named pPAF (for phoA fusion). The DNA fragment coding for the PhoA-LacZα fusion protein was obtained by overlap extension PCR of the set of fragments (the *phoA* fragment without the signal sequence and stop codon and the *lacZ*α fragment encoding amino acid residues 4 to 60 amplified from the chromosome of *E. coli* MG1655) (34). The *phoA-lacZ*α gene fragment was ligated into pPAF in place of the *phoA* gene fragment. The resulting plasmid was named pPLD (for *phoA-lacZ* dual reporter). The *rrnB* T1T2 coding sequence was amplified using pKK223-3 as a template (35). This fragment containing additional XbaI, HindIII, and KpnI sites (included in the forward primer) was inserted into the EcoRI and BamHI sites of pMC1403 to yield pMCT (for pMC1403 with terminators).

Construction of mini-Tn*10*::Tet^r insertion library. The stationary-phase cultures of BW25113 harboring pMC1403-*yebS* were infected with λ1098 (22) and incubated at 40°C on LB plates supplemented with Amp, Tet, and X-Gal for 16 h.

Assays for PhoA and LacZ activities. β-Galactosidase (BG [LacZ]) activity was assayed as described in reference 36. Alkaline phosphatase (AP [PhoA]) activity was assayed as described in reference 14 with slight modification. Briefly, to prevent the activation of AP localized in the cytoplasm, cultures were transferred to 1.5-ml microcentrifuge tubes containing iodoacetamide to yield a final concentration of 1 mM, and all buffers were supplemented with 1 mM iodoacetamide. Assays were conducted at 28°C. Normalized activities were calculated as the ratio of the absolute activity of a fusion to the strongest

TABLE 2 Plasmids used in this study[a]

Name	Feature(s)[b]	Reference or source	Parental plasmid	Oligonucleotide no.
pCP20	Amp^r Cm^r *flp*	40		
pBR322	Amp^r Tet^r	41		
pTrc99A	Amp^r	Pharmacia LKB Biotechnology		
pGADT7	Amp^r LEU2; GAL4AD fusion	BD Clontech		
pGBKT7	Km^r TRP2; GAL4BD fusion vector	BD Clontech		
pKK223-3	Amp^r	Pharmacia LKB Biotechnology		
pMC1403	Amp^r; translational *lacZ* fusion	42		
pBR322Δtet	pBR322 without shorter EcoRI-EcoRV region		pBR322	
pBR322-*pqiA*	pBR322 with P*pqiA*-*pqiA* coding sequence		pBR322	2, 13
pBR322-*pqiABC*	pBR322 with P*pqiA*-*pqiABC* coding sequence		pBR322	2, 36
pTrc99NK	pTrc99A without shorter NcoI-KpnI region		pTrc99A	45
pTrc99NK-*pqiABC*	pTrc99NK with *pqiABC* coding sequence		pTrc99NK	3, 36
pTrc99A-*pqiBC*(HA)	pTrc99A with *pqiBC* coding sequence		pTrc99A	25, 36
pTrc99A-*yebT*(HA)	pTrc99A with *yebT* coding sequence		pTrc99A	31, 32
pTcr99NK-*yebST*	pTrc99NK with *yebST* coding sequence		pTrc99NK	16, 32
pGADT7-*pqiB*	GAL4AD is fused with PqiB without its TM domain		pGADT7	27, 29
pGADT7-*pqiC*	GAL4AD is fused with PqiC without its lipobox		pGADT7	34, 37
pGBKT7-*pqiB*	GAL4BD is fused with PqiB without its TM domain		pGBKT7	27, 29
pGBKT7-*pqiC*	GAL4BD is fused with PqiC without its lipobox		pGBKT7	34, 37
pPAF	Translational PhoA fusion		pKK223-3	38, 39
pPAF-*pqiABymbA*	YmbA-PhoA fusion under the control of P*pqiA*		pPAF	1, 35
pPAF-*pqiABymbA* (BglII)	pPAF-*pqiABymbA* without the SoxS-binding site in P*pqiA*		pPAF-*pqiABymbA*	
pPAF-PqiA	PhoA fusions with PqiA and its C-terminally truncated fragments		pPAF	1, 4–12
pMCT	pMC1403 with *rrnB* T1T2		pMC1403	43, 44
pMCT-PqiA	LacZ fusions with PqiA and its C-terminally truncated fragments		pMCT	1, 4–12
pPLD	Translational PhoA-LacZα fusion		pPAF	38, 40, 41, 42
pPLD-P*lac*-YebS	PhoA-LacZα fusions with YebS and its C-terminally truncated fragments under the control of P*lac*		pPLD	46, 47, 48, 17–24
pPLD-PqiB	PqiB-PhoA-LacZα fusion under the control of *lacI*^{q}-P*trc* system		pPLD	25, 30, 49
pPLD-YebT	YebT-PhoA-LacZα fusion under the control of *lacI*^{q}-P*trc* system		pPLD	31, 33, 49
pMC1403-*yebS*	YebS′-′LacZ fusion under the control of P*yebS* (−480, +17)		pMC1403	14, 15

[a]Numbers of oligonucleotides used for each plasmid correspond with those in Table S1 in the supplemental material.
[b]Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Tet^r, tetracycline resistance; Km^r, kanamycin resistance.

absolute activity among the series. Then Ln (normalized AP activity/normalized BG activity) was calculated (17).

Protease accessibility assay. IPTG (0.1 mM) was added to a growing culture of BW25113 harboring a PqiC-HA-expressing vector, and this culture was incubated for an additional 1 h. The cells were collected by centrifugation, washed, and resuspended in Tris-sucrose buffer (10 mM Tris-HCl [pH 8.0], 250 mM sucrose) at a final concentration giving an optical density at 600 nm (OD_{600}) of 1.5. When necessary, 5 mM EDTA and 50 μg/ml proteinase K (Nacalai) were added. After incubation for 30 min at 25°C, 5 mM phenylmethanesulfonyl fluoride (PMSF) and an equal volume of 2× PAGE sample buffer were added, and then the samples were heated at 95°C for 5 min. SDS-PAGE was carried out under reducing conditions using 10% (wt/vol) gels. Proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5% (wt/vol) nonfat milk with phosphate-buffered saline–Tween 20 (PBS-T) (0.05% [vol/vol] Tween 20 in PBS) and then incubated in PBS-T containing antibodies (90 min at room temperature [RT] or overnight at 4°C). Monoclonal anti-HA mouse IgG antibody (Nacalai) and goat anti-mouse IgG antibody (Invitrogen) were diluted in the PBS-T at a 1:5,000 ratio. The membranes were washed with PBS-T using the following conditions: rinse three times, incubate for 15 min, and incubate for 5 min.

Yeast two-hybrid assays. SD medium has the above-listed ingredients with the appropriate supplement(s): histidine and leucine (no tryptophan), histidine (no tryptophan or leucine), or no additional supplement (no tryptophan, leucine, or histidine). GAL4BD fusion-expressing vectors were transformed into *S. cerevisiae* strain HF7c using the lithium acetate-polyethylene glycol (PEG) method (37). The transformants were spread on no-tryptophan plates and incubated at 30°C for 2 days. GAL4AD fusion-expressing vectors were transformed into HF7c harboring GAL4BD fusion-expressing vectors by the same procedure. The transformants were spotted on a no-tryptophan/no-leucine plate or a no-tryptophan/no-leucine/no-histidine plate and incubated at 30°C for 3 days (38).

Assay for SDS-EDTA sensitivity. Stationary-phase cultures of *E. coli* cells were serially diluted 10-fold in 0.85% NaCl solution, and 4 μl of each culture was spotted on an LB plate containing SDS and EDTA. These plates were then incubated at 37°C for 16 h.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00606-16.

TEXT S1, PDF file, 0.02 MB.

ACKNOWLEDGMENTS

We thank the National BioResource Project (NIG, Japan) for kindly supplying the *E. coli* strains used in this study. We thank Elizabeth Nakajima and Masafumi Funakoshi for critically reading and language editing the manuscript.

This work was financially supported in part by a Grant-in-Aid for Scientific Research (16K00545) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Q.-M. Zhang-Akiyama).

REFERENCES

1. Nikaido H. 1988. Bacterial resistance to antibiotics as a function of outer membrane permeability. J Antimicrob Chemother 22(Suppl A):17–22.
2. Maldonado RF, Sá-Correia I, Valvano MA. 2016. Lipopolysaccharide modification in Gram-negative bacteria during chronic infection. FEMS Microbiol Rev 40:480–493. https://doi.org/10.1093/femsre/fuw007.
3. Nikaido H. 2005. Restoring permeability barrier function to outer membrane. Chem Biol 12:507–509. https://doi.org/10.1016/j.chembiol.2005.05.001.
4. Malinverni JC, Silhavy TJ. 2009. An ABC transport system that maintains lipid asymmetry in the gram-negative outer membrane. Proc Natl Acad Sci U S A 106:8009–8014. https://doi.org/10.1073/pnas.0903229106.
5. Chitale S, Ehrt S, Kawamura I, Fujimura T, Shimono N, Anand N, Lu S, Cohen-Gould L, Riley LW. 2001. Recombinant *Mycobacterium tuberculosis* protein associated with mammalian cell entry. Cell Microbiol 3:247–254. https://doi.org/10.1046/j.1462-5822.2001.00110.x.
6. Casali N, Konieczny M, Schmidt MA, Riley LW. 2002. Invasion activity of a *Mycobacterium tuberculosis* peptide presented by the *Escherichia coli* AIDA autotransporter. Infect Immun 70:6846–6852. https://doi.org/10.1128/IAI.70.12.6846-6852.2002.
7. Gioffré A, Infante E, Aguilar D, Santangelo MP, Klepp L, Amadio A, Meikle V, Etchechoury I, Romano MI, Cataldi A, Hernández RP, Bigi F. 2005. Mutation in mce operons attenuates *Mycobacterium tuberculosis* virulence. Microbes Infect 7:325–334. https://doi.org/10.1016/j.micinf.2004.11.007.
8. Koh YS, Roe JH. 1996. Dual regulation of the paraquat-inducible gene *pqi-5* by SoxS and RpoS in *Escherichia coli*. Mol Microbiol 22:53–61. https://doi.org/10.1111/j.1365-2958.1996.tb02655.x.
9. Koh YS, Roe JH. 1995. Isolation of a novel paraquat-inducible (*pqi*) gene regulated by the *soxRS* locus in *Escherichia coli*. J Bacteriol 177:2673–2678.
10. Hassan HM, Fridovich I. 1979. Paraquat and *Escherichia coli*. Mechanism of production of extracellular superoxide radical. J Biol Chem 25:10846–10852.
11. Schmidt H, Hensel M. 2004. Pathogenicity islands in bacterial pathogenesis. Clin Microbiol Rev 17:14–56. https://doi.org/10.1128/CMR.17.1.14-56.2004.
12. Das A, Yanofsky C. 1989. Restoration of a translational stop-start overlap reinstates translational coupling in a mutant *trpB′-trpA* gene pair of the *Escherichia coli* tryptophan operon. Nucleic Acids Res 22:9333–9340.
13. Okuda S, Tokuda H. 2011. Lipoprotein sorting in bacteria. Annu Rev Microbiol 65:239–259. https://doi.org/10.1146/annurev-micro-090110-102859.
14. Derman AI, and Beckwith J. 1995. *Escherichia coli* alkaline phosphatase localized to the cytoplasm slowly acquires enzymatic activity in cells whose growth has been suspended: a caution for gene fusion studies. J Bacteriol 177:3764–3770.
15. Nilsson I, von Heijne G. 1990. Fine-tuning the topology of a polytopic membrane protein: role of positively and negatively charged amino acids. Cell 62:1135–1141. https://doi.org/10.1016/0092-8674(90)90390-Z.
16. Koch J, Guntrum R, Heintke S, Kyritsis C, Tampé R. 2004. Functional dissection of the transmembrane domains of the transporter associated with antigen processing (TAP). J Biol Chem 279:10142–10147. https://doi.org/10.1074/jbc.M312816200.
17. Tse YM, Yu M, Tsang JS. 2009. Topological analysis of a haloacid permease of a Burkholderia sp. bacterium with a PhoA-LacZ reporter. BMC Microbiol 9:233. https://doi.org/10.1186/1471-2180-9-233.
18. Korres H, Verma NK. 2004. Topological analysis of glucosyltransferase GtrV of *Shigella flexneri* by a dual reporter system and identification of a unique reentrant loop. J Biol Chem 279:22469–22476. https://doi.org/10.1074/jbc.M401316200.
19. Kovacs-Simon A, Titball RW, Michell SL. 2011. Lipoproteins of bacterial pathogens. Infec Immun 79:548–561. https://doi.org/10.1128/IAI.00682-10.
20. Tommassen J, Lugtenberg B. 1984. Amino terminus of outer membrane PhoE protein: localization by use of a *bla-phoE* hybrid gene. J Bacteriol 157:327–329.
21. Chong ZS, Woo WF, Chng SS. 2015. Osmoporin OmpC forms a complex with MlaA to maintain outer membrane lipid asymmetry in *Escherichia coli*. Mol Microbiol 98:1133–1146. https://doi.org/10.1111/mmi.13202.
22. Zhang QM, Ishikawa N, Nakahara T, Yonei S. 1998. *Escherichia coli* MutY protein has a guanine-DNA glycosylase that acts on 7,8-dihydro-8-oxoguanine:guanine mispair to prevent spontaneous G:C≥C:G transversions. Nucleic Acids Res 26:4669–4675. https://doi.org/10.1093/nar/26.20.4669.
23. Yethon JA, Vinogradov Perry EMB, Whitfield C. 2000. Mutation of the lipopolysaccharide core glycosyltransferase encoded by *waaG* destabilizes the outer membrane of *Escherichia coli* by interfering with core phosphorylation. J Bacteriol 182:5620–5623. https://doi.org/10.1128/JB.182.19.5620-5623.2000.
24. De Lay NR, Cronan JE. 2008. Genetic interaction between the *Escherichia coli* AcpT phosphopantetheinyl transferase and the YejM inner membrane protein. Genetics 178:1327–1337. https://doi.org/10.1534/genetics.107.081836.
25. Wu J, Weiss B. 1991. Two divergently transcribed genes, *soxR* and *soxS*, control a superoxide response regulon of *Escherichia coli*. J Bacteriol 173:2864–2871.
26. Pomposiello PJ, Bennik MH, Demple B. 2001. Genome-wide transcriptional profiling of the *Escherichia coli* responses to superoxide stress and sodium salicylate. J Bacteriol 183:3890–3902. https://doi.org/10.1128/JB.183.13.3890-3902.2001.
27. Martin RG, Rosner JL. 2002. Genomics of the *marA/soxS/rob* regulon of *Escherichia coli*: identification of directly activated promoters by application of molecular genetics and informatics to microarray data. Mol Microbiol 44:1611–1624. https://doi.org/10.1046/j.1365-2958.2002.02985.x.
28. Nakayama T, Yonekura S, Yonei S, Zhang-Akiyama QM. 2013. *Escherichia coli* pyruvate:flavodoxin oxidoreductase, YdbK—regulation of expression and biological roles in protection against oxidative stress. Genes Genet Syst 88:175–188. https://doi.org/10.1266/ggs.88.175.
29. Sutterlin HA, Shi H, May KL, Miguel A, Khare S, Huang KC, Silhavy TJ.

2016. Disruption of lipid homeostasis in the Gram-negative cell envelope activates a novel cell death pathway. Proc Natl Acad Sci U S A 113: 1565–1574. https://doi.org/10.1073/pnas.1601375113.

30. Lee JH, Lee KL, Yeo WS, Park SJ, Roe JH. 2009. SoxRS-mediated lipopolysaccharide modification enhances resistance against multiple drugs in *Escherichia coli*. J Bacteriol 191:4441–4450. https://doi.org/10.1128/JB.01474-08.
31. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2:2006.0008. https://doi.org/10.1038/msb4100050.
32. Doublet B, Douard G, Targant H, Meunier D, Madec JY, Cloeckaert A. 2008. Antibiotic marker modifications of lambda Red and FLP helper plasmids, pKD46 and pCP20, for inactivation of chromosomal genes using PCR products in multidrug-resistant strains. J Microbiol Methods 75:359–361. https://doi.org/10.1016/j.mimet.2008.06.010.
33. Inouye H, Barnes W, Beckwith J. 1982. Signal sequence of alkaline phosphatase of *Escherichia coli*. J Bacteriol 149:434–439.
34. Lee J, Shin MK, Ryu DK, Kim S, Ryu WS. 2010. Insertion and deletion mutagenesis by overlap extension PCR. Methods Mol Biol 634:137–146. https://doi.org/10.1007/978-1-60761-652-8_10.
35. Ghosh B, Grzadzielska E, Bhattacharya P, Peralta E, DeVito J, Das A. 1991. Specificity of antitermination mechanisms. Suppression of the terminator cluster T1-T2 of *Escherichia coli* ribosomal RNA operon, *rrnB*, by phage lambda antiterminators J Mol Biol 222:59–66. https://doi.org/10.1016/0022-2836(91)90737-Q.
36. Miller JH. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, NY.
37. Gietz RD. 2014. Yeast transformation by the LiAc/SS carrier DNA/PEG method. Methods Mol Biol 1205:1–12. https://doi.org/10.1007/978-1-4939-1363-3_1.
38. Miller J, Stagljar I. 2004. Using the yeast two-hybrid system to identify interacting proteins. Methods Mol Biol 261:247–262.
39. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645. https://doi.org/10.1073/pnas.120163297.
40. Cherepanov PP, Wackernagel W. 1995. Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 158:9–14. https://doi.org/10.1016/0378-1119(95)00193-A.
41. Bolivar F, Rodriguez RL, Betlach MC, Boyer HW. 1977. Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. Gene 2:75–93.
42. Casadaban MJ, Cohen SN. 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J Mol Biol 138:179–207. https://doi.org/10.1016/0022-2836(80)90283-1.