B1500, a small membrane protein, connects the two-component systems EvgS/EvgA and PhoQ/PhoP in *Escherichia coli*

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Two-component signal-transduction systems (TCSs) of bacteria are considered to form an intricate signal network to cope with various environmental stresses. One example of such a network in *Escherichia coli* is the signal transduction cascade from the EvgS/EvgA system to the PhoQ/PhoP system, where activation of the EvgS/EvgA system promotes expression of PhoP-activated genes. As a factor connecting this signal transduction cascade, we have identified a small inner membrane protein (65 aa), B1500. Expression of the *b1500* gene is directly regulated by the EvgS/EvgA system, and *b1500* expression from a heterologous promoter simultaneously activated the expression of *mgtA* and other PhoP regulon genes. This activation was PhoQ/PhoP-dependent and EvgS/EvgA-independent. Furthermore, deletion of *b1500* from an EvgS-activated strain suppressed *mgtA* expression. B1500 is localized in the inner membrane, and bacterial two-hybrid data showed that B1500 formed a complex with the sensor PhoQ. These results indicate that the small membrane protein, B1500, connected the signal transduction between EvgS/EvgA and PhoQ/PhoP systems by directly interacting with PhoQ, thus activating the PhoQ/PhoP system.

two-component signal transduction

The two-component signal transduction system (TCS) is the major system in bacteria for sensing environmental changes and transducing the information inside the cells to regulate gene expression (1). TCSs control the expression of genes for nutrient acquisition, virulence, antibiotic resistance, and numerous other pathways in diverse bacteria. TCS is composed of a membrane-bound sensor histidine kinase (HK) that perceives environmental stimuli and a cognate cytoplasmic response regulator (RR). The sensor HK monitors the environmental stimuli by autophosphorylating its conserved histidine residue, which in turn phosphorylates the conserved aspartate residue of its cognate response regulator. In most cases, RR binds to DNA regulatory sequences and affects transcription.

In *Escherichia coli*, 29 HKs, 32 RRs, and one histidine containing phosphotransfer (HPt) domain have been found by analyses of the *E. coli* K-12 genome (2). Oshima *et al.* proposed the existence of a network of functional interactions, such as cross-talks (transfer of phosphoryl groups from a sensory HK to a noncognate RR) and cascade signal transduction among multiple TCSs (3). Several examples of *in vivo* (4, 5, 6) and *in vitro* (7, 8) cross-talks, as well as signal transduction cascade between TCSs in *E. coli* (9–11), have been reported.

We reported signal transduction cascade between EvgS/EvgA and PhoQ/PhoP TCSs in *E. coli*. The EvgS/EvgA system confers acid resistance and multidrug resistance to *E. coli*, and it is very similar to the virulence-related BvgS/BvgA system in *Bordetella pertussis* (12, 13, 14), whereas the PhoQ/PhoP system responds to external Mg^{2+} and Ca^{2+} levels, regulating expression of the genes including Mg^{2+} transporters and LPS modification genes (15, 16). Cultivation of *E. coli* in neutral rich media results in activation of the PhoQ/PhoP system but not the EvgS/EvgA system. An addition of high concentration of Mg^{2+} turns off the PhoQ/PhoP system, presumably by activating the phosphatase activity of the sensor PhoQ (17). However, in the *evgS1* mutant strain, which constitutively activates the EvgS/EvgA system, the PhoQ/PhoP system remains active even at high Mg^{2+} levels. This signal transduction between the two TCSs can also occur as a result of overproduction of the EvgA regulator, which rules out phosphotransfer between the activated sensor EvgS and the noncognate regulator PhoP. Moreover, enhanced transcription of the *phoPQ* genes did not further activate expression of the PhoQ/PhoP regulated genes, and no EvgA binding consensus sequence (12, 18) was found in the promoter regions of the PhoP regulon genes. Thus, activation of the EvgS/EvgA system may increase or at least maintain the level of phospho-PhoP by (*i*) inhibiting the phosphatase activity of PhoQ, (*ii*) activating the PhoQ kinase, or (*iii*) protecting the phospho-PhoP against dephosphorylation by PhoQ.

It has been reported that some small (<200-aa) proteins regulate TCSs to adapt cells for rapid environmental changes. In *Salmonella enterica*, PmrD, a cytoplasmic protein composed of 85 aa, connects the PhoQ/PhoP and PmrB/PmrA TCSs (19). PmrD binds to the phosphorylated form of the response regulator PmrA at the N-terminal domain, thus preventing both its intrinsic dephosphorylation and that promoted by its cognate sensor kinase PmrB (20). Another small protein in *Salmonella* and in *E. coli* is IraP (86 aa), which enhances RpoS stability by interacting with MviA (*Salmonella*, 21) or RssB (*E. coli*, 22). Moreover, CpxP, a periplasmic protein (166 aa) of *E. coli*, interacts with the sensor domain of a histidine kinase CpxA to inhibit the CpxA/CpxR pathway (23).

In the present study, we have identified a regulatory protein, B1500, whose expression was enhanced by the EvgS/EvgA system; moreover, this protein activated the PhoQ/PhoP system, thus connecting the two TCSs. B1500, composed of 65 aa, is localized in the inner membrane and forms a complex with the sensor PhoQ. Therefore, B1500 directly interacts with PhoQ to activate the PhoQ/PhoP system. To our knowledge, this is the first report of such a small membrane protein connecting two TCSs.

Results

Cloning and Sequencing of a Gene Involved in Signal Transduction Between EvgS/EvgA and PhoQ/PhoP Systems. We set up a screening system to identify any additional factor(s) involved in signal trans-

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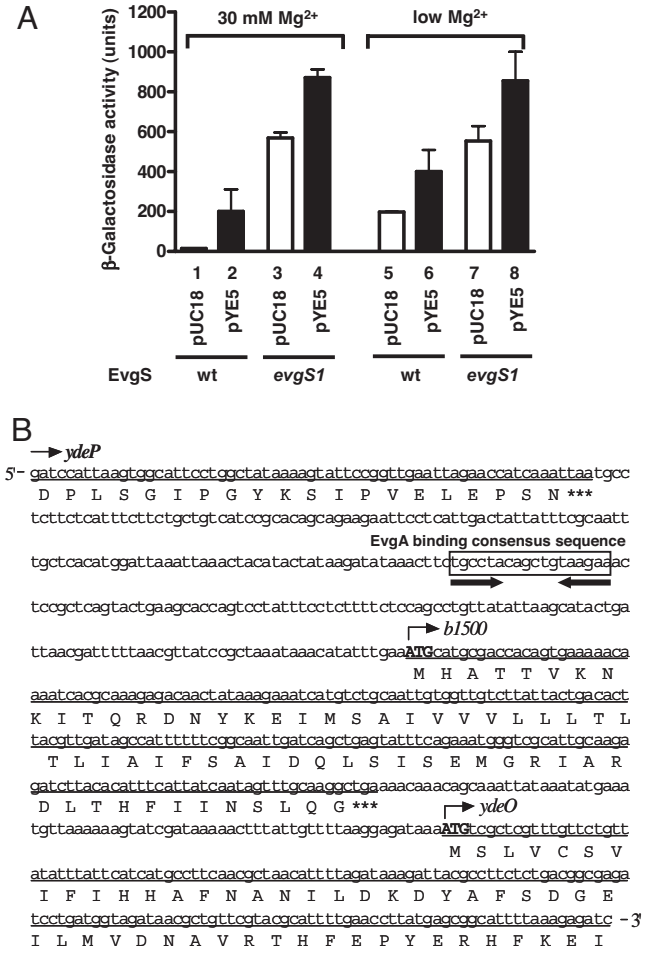

Fig. 1. Characterization of pYE5 plasmid obtained from shotgun screening. (*A*) Transcriptional activity of *mgtA* in MG1601/pUC18, MG1601/pYE5, MG1601*evgS1*/pUC18, and MG1601*evgS1*/pYE5 strains in the presence and absence of 30 mM $MgCl_2$. (*B*) Nucleic acid sequence of the insert portion of pYE5 corresponds to AE000247 (complementary, 1581559–1582293). ORFs (underlined) included in the insert were the C-terminal part of *ydeP*, the complete *b1500*, and the N-terminal part of *ydeO*. Corresponding amino acids are shown under the line, and the stop codons are shown as ***. The EvgA binding consensus sequence (18) is boxed, and the inverted repeat is indicated by the bold arrows.

duction between the EvgS/EvgA and PhoQ/PhoP systems. Genomic DNA from MA204, a strain deleted for *evgA*, was partially digested with *Sau*IIIAI and cloned into pUC18. This plasmid library was then transformed into MG1601, an *E. coli* strain with a *lacZ* gene under control of the *mgtA* promoter (a PhoP regulated gene) and selected for ampicillin and kanamycin resistance in the presence of X-gal and 100 mM $MgCl_2$. At such a high concentration of Mg^{2+}, the PhoQ/PhoP system is usually inactivated and *mgtA* expression is repressed. From this screening, we identified one positive plasmid, pYE5. As shown in Fig. 1*A*, MG1601/pYE5 grown at 30 mM Mg^{2+} concentration retained high *mgtA* transcriptional activity (column 2), whereas the control strain, MG1601/pUC18 at high Mg^{2+} concentration, showed suppressed expression (column 1). MG1601/pYE5 also showed higher *mgtA* transcriptional activity than MG1601/pUC18 at low Mg^{2+} concentration (columns 5 and 6).

Sequencing of the insert region of pYE5 revealed the C-terminal portion of *ydeP*, the complete ORF of *b1500*, and the N-terminal portion of *ydeO* (Fig. 1*B*). All three of these genes have been reported to be directly regulated by EvgA, and *b1500* composes an operon with *ydeO* (18). The gene *ydeP* codes for a putative oxidoreductase, and *ydeO* codes for a XylS/AraC-type regulatory protein (24). Overexpression of *ydeP* and *ydeO* confers acid resistance to *E. coli* (25), but the function of *b1500* is unknown. In addition, an EvgA binding consensus sequence is present in the *b1500*/*ydeO* promoter (18) (Fig. 1*B*). Footprinting analysis showed that EvgA indeed bound directly to the promoter region of *b1500* [supporting information (SI) Fig. 7]. Switching the host strain of MG1601/pYE5 to an EvgS-active mutant (MG1601*evgS1*) increased *mgtA* transcriptional activity at both low and high Mg^{2+} concentration (Fig. 1*A*, columns 4 and 8).

The *b1500* Gene Is Involved in Signal Transduction Between EvgS/EvgA and PhoQ/PhoP. Because *b1500* is the only gene in pYE5 that encodes a complete ORF, we hypothesized that *b1500* may contribute to signal transduction between the EvgS/EvgA and PhoQ/PhoP systems. The wild-type strain and *b1500*-deleted strain showed repressed *mgtA* expression at high concentration of Mg^{2+} (Fig. 2 *A* and *B*, columns 1 and 2). When the EvgS/EvgA system was activated by the *evgS1* mutation, *mgtA* expression was enhanced (Fig. 2 *A* and *B*, column 3) as reported (10). However, *b1500*-deleted *evgS1* strain did not induce *mgtA* expression above the wild type level (Fig. 2 *A* and *B*, column 4). Furthermore, complementation of the MG1655*evgS1 b1500* strain with pYE5 rescued the *b1500* deletion (Fig. 2 *A* and *B*, column 8), whereas MG1655, MG1655 *b1500*, MG1655 *evgS1* with pYE5 also enhanced *mgtA* expression (Fig. 2 *A* and *B*, columns 5, 6, and 7).

Next, the *b1500* ORF was expressed as a His-tag fusion protein in MG1601 and its *phoQ*-, *phoP*-, *evgS*-, and *evgA*-deleted strains. In the presence of 30 mM $MgCl_2$, *mgtA* transcription was activated by expression of *b1500* in MG1601 (Fig. 2*C*, column 2). In contrast, expression of *b1500* had no effect in *phoQ* and *phoP* deleted strains (Fig. 2*C*, columns 4 and 6), which indicated that the transcriptional activation of *mgtA* by *b1500* was PhoQ/PhoP-dependent. However, deletion of neither *evgS* nor *evgA* suppressed the activation of *mgtA* transcripton by *b1500* (Fig. 2*C*, columns 8 and 10). Therefore, the transcriptional activation of *mgtA* was not due to the activation of the EvgS/EvgA system by *b1500* but to a PhoQ/PhoP-dependent pathway.

In our previous study (10), activation of the EvgS/EvgA system promoted transcriptional activity of PhoP regulated genes. Thus, we examined the effect of *b1500* on transcriptional activities of four genes directly regulated by PhoP, namely, *phoPQ*, *mgtA*, *mgrB*, and *rstAB* (16) by S1 nuclease assay. As shown in Fig. 3, the presence of 30 mM $MgCl_2$ repressed expression of these four genes (lanes 1 and 2). However, *b1500* promoted expression of these four genes even in the presence of 30 mM $MgCl_2$ (lanes 3 and 4). This indicated that *b1500* promoted not only transcription of the *mgtA* gene, but also those of the other PhoP regulated genes, meaning that the PhoQ/PhoP system was activated. The genes, *slyB*, *yrbL* and *hemL*, which are also directly regulated by PhoP (16), were also activated by *b1500* (SI Fig. 8). No apparent effect on transcription of the investigated promoters dependent on addition of isopropyl β-D-thiogalactopyranoside (IPTG) was observed, suggesting that only a small amount of B1500 was sufficient to activate the PhoQ/PhoP system.

The *b1500* Gene Encodes a Small Membrane Protein. The ORF, *b1500*, is only 198 bp long, and it is predicted to encode for a 65-aa protein. A BLASTP search of B1500 identified homologous ORFs in four *Shigella* species; *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*, and *Shigella dysenteriae*. Using MEMSTAT (26) and HMMTOP 2.0 (27) programs for topology predictions, we predicted that amino acids 19–40 may adopt a transmembrane domain structure (Fig. 4*C*).

To determine whether B1500 localized to the membrane, we overexpressed B1500 in MG1601, lysed the exponential phase cells by sonication, briefly centrifuged them to remove the cell debris and

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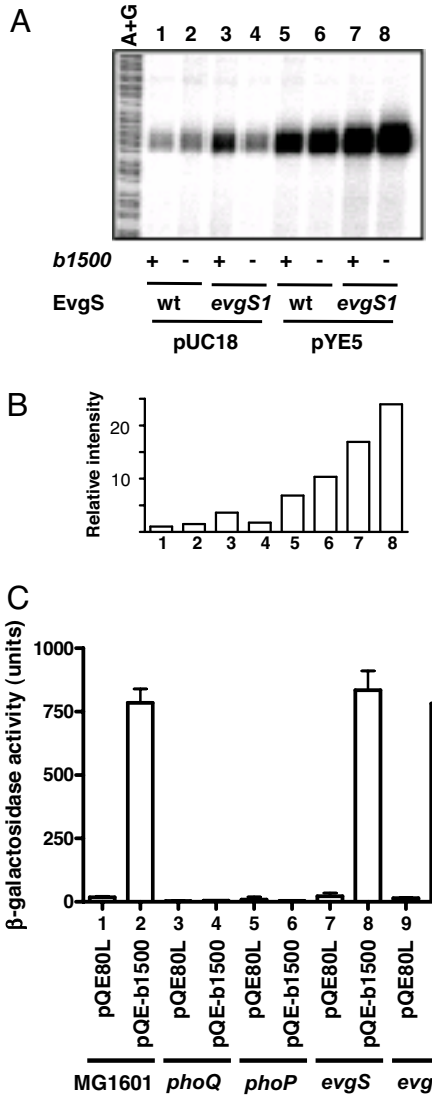

Fig. 2. Functional analysis of *b1500*. (*A*) Deletion of *b1500* suppresses *mgtA* transcription. S1 nuclease assay of *mgtA* was performed against the following strains grown in the presence of 30 mM $MgCl_2$: lanes 1, MG1655/pUC18; lane 2, MG1655 *b1500*/pUC18; lane 3, MG1655 *evgS1*/pUC18; lane 4, MG1655 *evgS1 b1500*/pUC18; lane 5, MG1655/pYE5; lane 6, MG1655 *b1500*/pYE5; lane 7, MG1655 *evgS1*/pYE5; and lane 8, MG1655 *evgS1 b1500*/pYE5. A+G, Maxam–Gilbert sequencing ladder. (*B*) Radioactivity intensity of each lane in *A* relative to the radioactivity of lane 1. (*C*) Overexpression of *b1500* promotes *mgtA* transcription in a PhoQ/PhoP-dependent and EvgS/EvgA-independent manner. Column 1, MG1601/pQE80L; column 2, MG1601/pQE-b1500; column 3, MG1607/pQE80L; column 4, MG1607/pQE-b1500; column 5, MG1622/pQE80L; column 6, MG1622/pQE-b1500; column 7, MG1601 *evgS*/pQE80L; column 8, MG1601 *evgS*/pQE-b1500; column 9, MG1601 *evgA*/pQE80L; and column 10, MG1601 *evgA*/pQE-b1500. Cells were grown in the presence of 30 mM $MgCl_2$. For induction of *b1500*, IPTG, at a final concentration of 1 mM, was added to the culture at OD_{600} = 0.4.

insoluble proteins, and ultracentrifuged the supernatant to obtain membrane fractions. To these fractions, Western blot analysis was performed by using an anti-His-tag antibody to detect the expressed His-tagged B1500 protein. As shown in Fig. 4*A*, B1500 was detected in the pellet after ultracentrifugation, indicating that it localized to the membrane. We next treated the membrane fraction with 0.1, 0.2, 0.3, and 0.4% sarcosyl and ultracentrifuged them to separate the inner (supernatant) and outer membrane (pellet). The majority of B1500 was detected from the inner membrane fraction, indicating that it localized to the inner membrane (Fig. 4*B*).

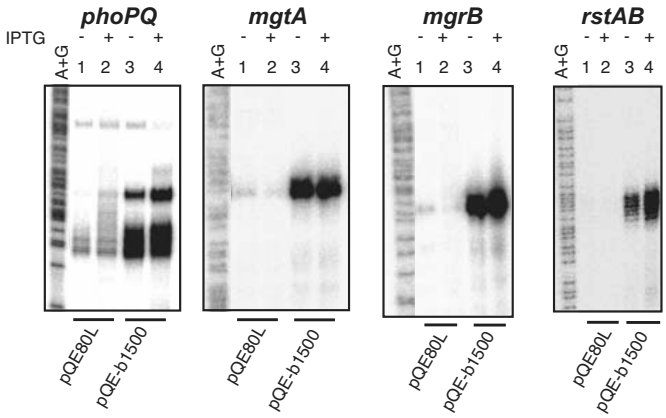

Fig. 3. Transcriptional activation of the PhoP regulon by *b1500*. S1 nuclease assay of *phoPQ*, *mgtA*, *mgrB* and *rstAB* was performed against cells grown in the presence of 30 mM $MgCl_2$, with and without the addition of 1 mM IPTG at OD_{600} = 0.4. Lanes 1 and 2 are MG1601/pQE80L and Lanes 3 and 4 are MG1601/pQE-b1500.

B1500 and PhoQ Interact in a Two-Hybrid Assay. Given the fact that B1500 resided in the inner membrane, the most likely target of B1500 would be the sensor PhoQ. To investigate whether B1500 and PhoQ interact with each other, we used a bacterial two-hybrid system (28), which was recently applied to show direct interaction between membrane proteins of *Bacillus subtilis* (29). In this system, the proteins of interest are genetically fused to two fragments (T25 and T18) of the catalytic domain of *B. pertussis* adenylate cyclase and coexpressed in an *E. coli* deficient in endogenous adenylate cyclase. Interaction of the two hybrid proteins results in a functional complementation between the T25 and T18 fragments, which in turn relaxes catabolite repression and induces expression of *lacZ* among others. Hence, interaction of the two proteins can be studied by measuring the β-galactosidase activity.

As demonstrated in Fig. 5, coexpression of T25-B1500 with PhoQ-T18 resulted in β-galactosidase activity that was 23-fold over the background (T25/T18), indicating that B1500 and PhoQ directly interacted with each other. Because the T25 fragment was fused to the N-terminal end of B1500, the N-terminal end of B1500 had to be exposed on the cytoplasmic side for the functional complementation between T25 and T18. Thus, according to the classification method suggested by S. J. Singer (30), B1500 is classified as a Type II membrane protein, because it spans the membrane once but has its N terminus on the cytoplasmic side of the cell and the C terminus on the exterior. This topology agrees with the "positive inside rule" (31), because four positively charged amino acids are clustered at the N-terminal end of B1500 (Fig. 4*C*). High β-galactosidase activity (Fig. 5) was also observed from a strain expressing T25-B1500 and T18c-B1500 (the T18 fragment was fused to the N-terminal end of B1500), suggesting that B1500 formed oligomers.

Discussion

The *b1500* gene found by our genetic screening is located between the *ydeP* and *ydeO* genes (Fig. 1*B*). Nadler *et al.* (32) recently reported that YdeP and YdeO act conjointly to repress expression of the type III secretion system (TTSS) genes of enteropathogenic *E. coli*. When they overexpressed either *ydeP* or *ydeO*, TTSS protein production decreased. In contrast, overexpression of *b1500* did not affect TTSS protein production. To date, no function has been assigned to *b1500*. In fact, an analysis performed in the 2005 *E. coli* annotation update (33) suggested that this gene did not exist in *E. coli* K-12, and therefore it was converted to an instance of the phantom genes class, implying that it is no longer considered an actual gene. However, in the present study, we have identified the gene, *b1500*, whose expression is directly induced by the EvgS/EvgA

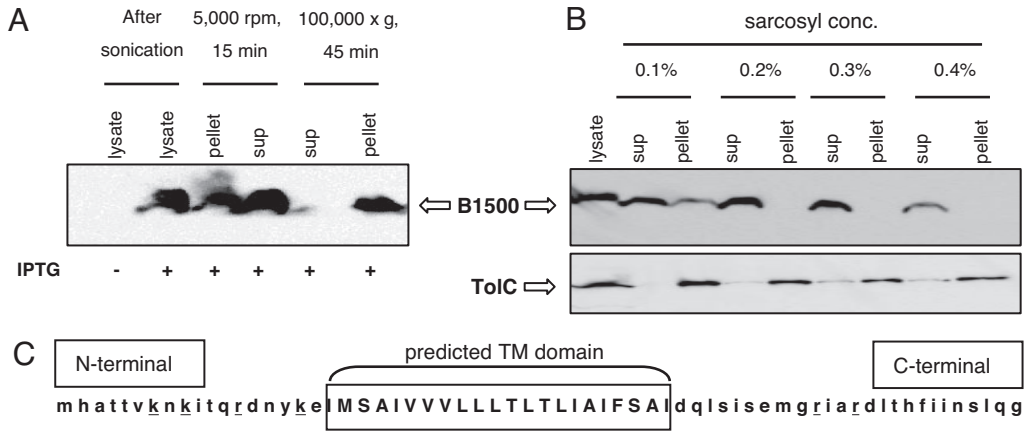

Fig. 4. B1500 is localized in the inner membrane. (*A*) Western blot analysis against anti-His tag antibody for B1500 detection. MG1601/pQE-b1500 was grown in the presence of 30 mM $MgCl_2$, and IPTG, at a final concentration of 1 mM, was added to the culture at OD_{600} = 0.4 for induction of B1500. Cells were lysed by sonication, followed by brief centrifugation and ultracentrifugation. (*B*) Sarcosyl treatment of the membrane fraction. Total membrane fraction after ultracentrifugation was treated with sarcosyl at different concentrations, followed by ultracentrifugation for the separation of inner and outer membrane. Anti-TolC antibody was used for the detection of TolC, an outer membrane protein control. (*C*) Predicted topology of B1500. Bold letters indicate the transmembrane domain predicted by the programs MEMSTAT and HMMTOP. Lysines and arginines are underlined.

system, and expression of this gene from a heterologous promoter simultaneously activated the expression of PhoP regulon genes in a PhoQ/PhoP-dependent manner. These results suggest that *b1500* is involved in signal transduction between EvgS/EvgA and PhoQ/PhoP TCSs, thus, associating a novel function with this small ORF.

As demonstrated in Fig. 4*B*, B1500 localized to the inner membrane. Membrane proteins such as NlpE (236 aa) and RcsF (134 aa), which are relatively small lipoproteins in the outer membrane of *E. coli*, activate the histidine kinases CpxA and RcsC, respectively (34, 35, 36). Although larger in size, YycH (458 aa) of *B. subtilis* is located external to the cell membrane and has one transmembrane domain at its N-terminal. Considering the location of this protein and the results of bacterial two-hybrid studies between YycH and the sensor YycG histidine kinase, YycH has been suggested to suppress the YycG/YycF system by direct interaction with the sensor domain of YycG (29, 37). Another example is that overproduction of DjlA (271 aa), an inner membrane protein belonging to the DnaJ family of *E. coli*, also activates the sensor RcsC (38). All of these membrane proteins interact with the histidine kinase sensor and not with the response regulator of TCSs. From our bacterial two-hybrid assay, we have found that B1500 also interacted with the sensor PhoQ to activate the PhoQ/PhoP system. Whether this activation results from inhibition of the phosphatase activity of PhoQ or from activation of the kinase activity of PhoQ remains to be investigated.

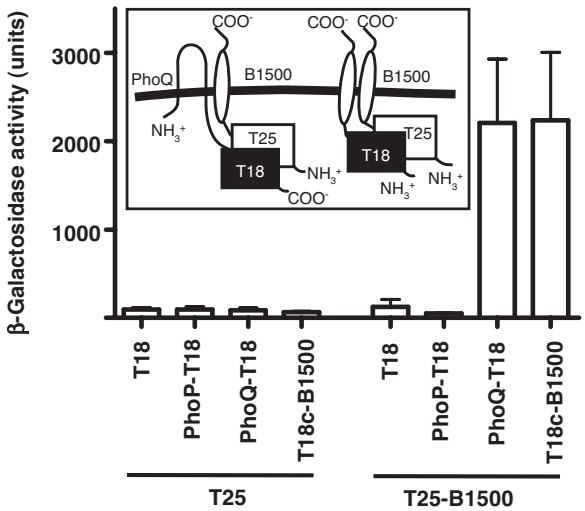

Fig. 5. A bacterial two-hybrid assay reveals interaction between B1500 and PhoQ. β-Galactosidase activity was determined for adenylate cyclase-deficient *E. coli* strain, DHP1, harboring plasmids expressing *B. pertussis* adenylate cyclase fragment T18 and T25 either unfused or fused to *b1500*, *phoP*, and *phoQ*. β-Galactosidase activity above background levels indicates an interaction between the coexpressed hybrid constructs. Column 1, pT25/pT18; column 2, pT25/pT18-PhoP; column 3, pT25/pT18-PhoQ; column 4, pT25/pT18c-B1500; column 5, pT25-B1500/pT18; column 6, pT25-B1500/pT18-PhoP; column 7, pT25-B1500/pT18-PhoQ; and column 8, pT25-B1500/pT18c-B1500.

The EvgS/EvgA TCS is involved in acid resistance. Acid resistance in exponentially growing cells is induced by the overexpression of EvgA (18, 25, 39) and the activation of the sensor EvgS (unpublished results). The phosphorylated EvgA induces the *b1500-ydeO* operon, and the transcriptional regulator, YdeO, sequentially induces another transcriptional regulator, GadE. It has been reported that EvgA also directly induces GadE expression, forming a branched pathway of regulation (40). Subsequently, GadE directly up-regulates glutamate-dependent acid resistance genes, *gadAX* and *gadBC*, as well as *hdeA*, a periplasmic chaperone gene protecting the cell from organic acid stress, and *hdeD*, a putative membrane protein gene participating in acid resistance exhibited only at high cell densities (18, 40, 41, 42). These data indicate that the EvgS/EvgA system regulates the expression of acid resistance genes (Fig. 6).

Interestingly, the PhoQ/PhoP system also confers acid resistance. In a recent report, Zwir *et al.* (43) verified that PhoP played a critical role in the control of acid resistance: Transcription of *hdeA* and *gadAX* is induced at low Mg^{2+} in a PhoP- and YhiW (GadW)-dependent fashion (Fig. 6). These results indicate that both EvgS/EvgA and PhoQ/PhoP are involved in the expression of acid resistance genes. As shown by Masuda *et al.* (18), induction of the *b1500-ydeO* operon triggered acid resistance by YdeO, however, whether *b1500* was involved in acid resistance had remained undetermined. In our recent study, deletion of *b1500* from an EvgS-activated strain reduced the acid-resistant phenotype conferred by EvgS activation. Moreover, deletion of *phoP* or *phoQ* from an EvgS-activated strain also reduced such acid resistant phenotype (unpublished results). These results indicate that acid resistance induced by the activation of EvgS was strongly dependent on the PhoQ/PhoP system, suggesting a biological role for B1500.

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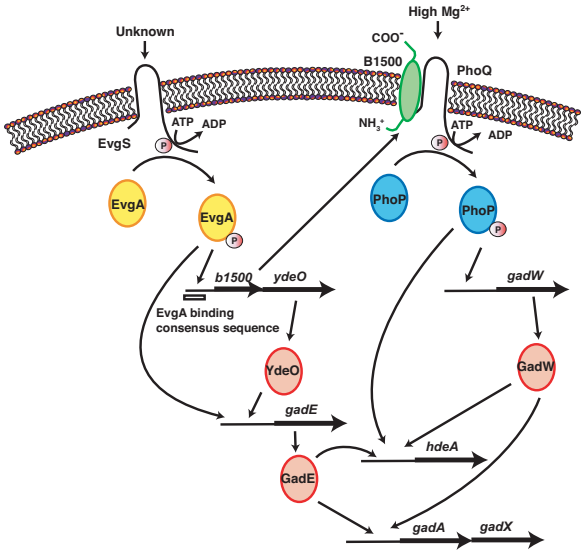

Fig. 6. A model of acid resistance controlled by EvgS/EvgA and PhoQ/PhoP TCSs connected by B1500.

In conclusion, the present study identified B1500 as a factor connecting the EvgS/EvgA and PhoQ/PhoP systems by directly interacting with PhoQ and increasing the level of PhoP phosphorylation. EvgS is reported to sense acidic pH in minimal media (40) and PhoQ of *Salmonella* also senses low pH by changing its conformation (44). By connecting the two systems, an intricate regulation network of acid resistance genes as shown in Fig. 6 is formed, which may help the cells for better adaptation to the acidic environment.

Materials and Methods

Bacterial Strains and Growth Conditions. The *E. coli* strains used in this study are listed in Table 1. The *phoP* strain (MG1622) and *phoQ* strain (MG1607) were constructed with P1 transduction from the donors WP3022 and WQ3007 (16) to the recipient MG1601. Similarly, the *evgA* strain (MG1601 *evgA*) and *evgS* strain (MG1601 *evgS*) were constructed with P1 transduction from the donors MC204 and CS7 to the recipient MG1601. Deletion of *b1500* was carried out by the method of Datsenko and Wanner (45). Primers b1500-H1 (5′-ACTGATTAACGATTTTTAACGTTATCCGCTAAATAAACATATTTGAAATGATTCCGGG GATCCGTCGACC-3′) and b1500-H2 (5′-ATTTCATATTTATAATTTGCTGTTTGTTTTCAGCC TTGCAAACTATTGATTGTAGGCTGGAGCTGCTTCG-3′) were used to delete the *b1500* of strain BW25113. This BW25113 *b1500* strain was used as the donor strain for P1 transduction to construct MG1655 *b1500*. The *evgS1* mutants of MG1601, MG1655 and MG1655 *b1500* were constructed by two consecutive P1 transductions with CS7 and KMR2001 as the donor strains.

Bacteria were grown at 37° C in a Luria-Bertani (LB) medium [1% Bacto tryptone (Difco), 0.5% Bacto yeast extract (Difco), and 1% NaCl, pH 7.5]. $MgCl_2$ was added to a final concentration of 30 mM when necessary. For induction of the T5 promoter of pQE-b1500, IPTG at a final concentration of 1 mM was added to the culture at OD_{600} of 0.4. Ampicillin, chloramphenicol, kanamycin, and tetracycline were used at 100 μg/ml, 25 μg/ml, 25 μg/ml, and 12.5 μg/ml, respectively.

Shotgun Screening. A cloning vector, pUC18 (Nippon Gene), was digested with BamHI (Toyobo, Japan) and treated with alkaline phosphatase (calf intestine phosphatase; Nippon Gene). Genome DNA from MA204 (MC4100 *evgA*) was briefly digested with Sau3AI (Toyobo). The digested fragments larger than 1,000 bp were ligated to the BamHI-digested pUC18 and transformed to MG1601. Positive-blue colonies were selected on LB agar plates supplemented with 0.008% X-gal, 100 mM $MgCl_2$, kanamycin, and ampicillin. Positive colonies were picked and amplified, and their plasmids were recovered. These plasmids were retransformed into MG1601 and spread onto the same supplemented LB agar plates for selection. The positive clones from this second selection were cultured in LB supplemented with 30 mM $MgCl_2$, kanamycin, and ampicillin, and their β-galactosidase activity was assayed for the third selection. Plasmids were recovered from the final positive clones. Nucleic acid sequence of the insert region of these plasmids were performed by ABI3100 (Applied BioSystems) with pUC19F (5′-GGATGTGC TGCAAGGCGATTAAGTTGGGTA-3′) and pUC19R (5′-GTATGTTGTGTGGAATTGTGAGCG GATAAC-3′) primers, and a BLAST homology search of the acquired sequence was carried out.

Plasmid Construction. The ORF of *b1500* was amplified by PCR with pYE5 as the template and primers b1500FBamHI (5′-AAACATATCG*GGATCC*CGTGCGACCACAGT-3′) and b1500RHindIII (5′-TATTCCC*AAGCTT*GGGTTTGTTTTCAGCCT-3′). The PCR product was digested with BamHI and

Table 1. Bacterial strains used in this study

Strain	Description	Ref. or source
MG1655	Wild-type *E. coli*	
MA204	MC4100 *evgA*::*kan*	Laboratory stock
MC204	MC4100 *evgA*::*cat*	Laboratory stock
CS7	JC7623 *evgS*::*cat*	12
MG1601	MC4100 *mgtA*:: *placMu55*	15
MG1607	MG1601 *phoQ*::*cat*	15
MG1622	MG1601 *phoP*::*cat*	15
MG1601 *evgS*	MG1601 *evgS*::*cat*	This study
MG1601 *evgA*	MG1601 *evgA*::*cat*	This study
BW25113 *b1500*	BW25113 *b1500*::*kan*	This study
MG1655 *b1500*	MG1655 *b1500*::*kan*	This study
KMR2001	KMY2001 *fadL71*::*Tn10*	12
MG1601 *evgS1*	MG1601 *evgS1 fadL71*::*Tn10*	This study
MG1655 *evgS1*	MG1655 *evgS1 fadL71*::*Tn10*	This study
MG1655 *evgS1 b1500*	MG1655 *evgS1 fadL*::*Tn10 b1500*::*kan*	This study
DHP1	F$^-$ *glnV44(AS) recA1 endA1 gyrA96 (Nal^r) thi1 hsdR17 spoT1 rfbD1 cya$^-$*	28

HindIII (Toyobo) and then ligated into BamHI and HindIII sites of pQE80L (Qiagen). The inserted region of the constructed plasmid was confirmed by sequencing, and the plasmid was named pQE-*b1500*. The amino acid sequence of the N-terminal region of the protein expressed from this plasmid is MRGSHHHHHHGSR. This sequence is fused with a B1500 fragment starting from the third amino acid (Ala) to the stop codon.

Bacterial two-hybrid vectors were created for PhoP, PhoQ, and B1500. The respective coding sequences were PCR-amplified introducing restriction sites, using the primers listed in SI Table 2. The PCR products were digested with respective restriction enzymes and cloned into plasmids, pT18, and pT25, resulting in pT18-PhoP, pT18-PhoQ, and pT25-B1500. The pT18 constructs expressed the respective genes fused to the N terminus of the *B. pertussis* adenylate cyclase gene fragment T18, and the pT25 constructs fused to the C terminus of fragment T25 (28). To fuse B1500 to the C terminus of fragment T18, the stop codon of T18 in pT18 was first deleted by ligating the PCR fragment amplified by primers T18F-KpnI and T18R-BamHI to pT18. This plasmid was named pT18c. A PCR-amplified fragment of *b1500* digested with BamHI and NotI was cloned into the 3′ end of the T18 gene fragment of pT18c, resulting in pT18c-B1500.

β-Galactosidase Assay. Cells grown to OD_{600} of 0.7–0.9 at 37° C in LB medium with appropriate supplements were subjected to β-galactosidase assay and expressed as Miller units (46). The data shown are means and standard deviations from three individual cultures.

RNA Preparation and S1 Nuclease Assay. Total RNA was extracted with hot phenol from a mid-exponential-phase culture (OD_{600} 0.7–0.9) at 60° C (47). S1 nuclease assay was carried out as described by Kato *et al.* (12). In brief, ^{32}P-end-labeled probe was prepared by PCR, using the primers listed in SI Table 3, MC4100 genomic DNA as the template, and ExTaq DNA polymerase (Takara). A mixture of ^{32}P-end-labeled probe and 100 μg of total RNA was incubated for 10 min at 75° C, then gradually cooled to 37° C and incubated overnight for hybridization, and finally subjected to S1 nuclease (Takara) digestion for 10 min at 37° C. Undigested RNA-probe DNA was extracted with phenol, precipitated with ethanol, and subjected to electrophoresis on a 6% (weight/volume) polyacrylamide sequencing gel containing 8 M urea. The radioactivity was measured by BAS1000 Mac (Fuji Film) and analyzed with MacBAS2.2 (Fuji Film).

Membrane Preparation and B1500 Detection. Strain pQE-*b1500*/MG1601 was grown in an LB medium with appropriate supplements to a mid-exponential-phase (OD_{600} 0.7–0.9). Cells were collected by centrifugation, and resuspended in lysis buffer [50 mM Tris·HCl (pH 8.0), 100 mM NaCl] with 1 mM PMSF. After sonication, the lysates were centrifuged for 10 min at 2,300 × *g*, 4° C, to remove cell debris and insoluble proteins. The supernatant was centrifuged again for 45 min at 100,000 × *g*, 4° C. The supernatant was collected as the cytoplasmic fraction, and the pellet was collected as the membrane fraction. For separation of the membrane to inner and outer membrane fractions, the membrane fraction was treated with 0.1, 0.2, 0.3, and 0.4% sarcosyl (N-lauroylsarcosine sodium salt solution; Fluka) for 1 h at room temperature followed by centrifugation for 45 min at 100,000 × *g*, 4° C. All of the samples were adjusted to equal final volume for comparison. Then, 10 μl of the samples were separated by SDS/PAGE, and the proteins were then transferred to a polyvinylidene difluoride membrane (Immobilon-P transfer membranes; Millipore) and probed with anti-His-tag antibody (GE Healthcare) or anti-TolC antibody (48). Detection was carried out with ECL Peroxidase-labeled anti-mouse antibody for anti-His tag antibody, ECL Peroxidase-labeled anti-rabbit antibody for anti-TolC antibody, and ECL Plus solution (ECL Plus Western blotting detection system; GE Healthcare). The results were analyzed by ImageQuant 400 (GE Healthcare).

Bacterial Two-Hybrid Assay. The bacterial two-hybrid assay was performed essentially as described in ref. 28. Here, pT25-derived plasmids and pT18- or pT18c-derived plasmids were cotransformed into the adenylate cyclase-deficient *E. coli* strain, DHP1. Transformants were selected by using chloramphenicol and ampicillin. To quantify the interaction, β-galactosidase activity of mid-exponential-growth cells were measured.

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