JB Journals.ASM.org

Alkali Metals in Addition to Acidic pH Activate the EvgS Histidine Kinase Sensor in *Escherichia coli*

Yoko Eguchi, Ryutaro Utsumi

Department of Bioscience, Graduate School of Agriculture, Kinki University, Nakamachi, Nara, Japan

Two-component signal transduction systems (TCSs) in bacteria perceive environmental stress and transmit the information via phosphorelay to adjust multiple cellular functions for adaptation. The EvgS/EvgA system is a TCS that confers acid resistance to *Escherichia coli* cells. Activation of the EvgS sensor initiates a cascade of transcription factors, EvgA, YdeO, and GadE, which induce the expression of a large group of acid resistance genes. We searched for signals activating EvgS and found that a high concentration of alkali metals (Na^+, K^+) in addition to low pH was essential for the activation. EvgS is a histidine kinase, with a large periplasmic sensor region consisting of two tandem PBPb (bacterial periplasmic solute-binding protein) domains at its N terminus. The periplasmic sensor region of EvgS was necessary for EvgS activation, and Leu152, located within the first PBPb domain, was involved in the activation. Furthermore, chimeras of EvgS and PhoQ histidine kinases suggested that alkali metals were perceived at the periplasmic sensor region, whereas the cytoplasmic linker domain, connecting the transmembrane region and the histidine kinase domain, was required for low-pH perception.

Bacteria adapt efficiently to the fluctuating environments that they encounter. Sensing the extracellular/intracellular environmental changes as signals and transducing the information for appropriate changes in gene expression and protein activity are essential for their adaptation. Two-component signal transduction systems (TCSs), typically composed of a signal-perceiving sensor histidine kinase (HK) and its cognate response regulator (RR), are often utilized by the bacteria for responding to environmental changes. Upon signal perception, a HK autophosphorylates its conserved histidine residue and transfers the phosphate to its cognate RR. The phosphorylated RR regulates gene expression or modifies protein activity, thus imparting environmental fitness to the cell. Some TCSs are essential for pathogenic bacteria to adapt themselves to the environment inside the host and to elicit pathogenicity. This is shown by the presence of a number of virulence-related TCSs in pathogens (1).

The neutrophilic bacterium *Escherichia coli* has evolved a complicated network of acid resistance (AR) (2). Its acid resistance helps it to pass through the extremely acidic barrier of the stomach to reach the intestine. *E. coli* possesses at least four AR systems to combat the acid barrier. AR system 1 is repressed by glucose, dependent on RpoS, and operative during pH 2.5 challenges in minimal medium without the addition of exogenous molecules. The other three AR systems require the addition of glutamic acid (AR system 2), arginine (AR system 3), or lysine (AR system 4). These systems utilize the amino-acid-dependent decarboxylase/antiporter systems (2). Among the four systems, the most effective is AR system 2, which relies on the action of two glutamic acid decarboxylase isozymes, GadA and GadB, and also the glutamate/γ-amino butyric acid (GABA) antiporter GadC. GadA and GadB are pyridoxal phosphate-containing enzymes that replace the α-carboxyl group of glutamate with a proton from the cytoplasm. The reaction consumes protons, thus reducing the intracellular proton level. The end products are CO_2 and GABA, and GadC expels GABA in exchange for importing glutamate (3–5). This system is under extremely complicated control, and at least 10 regulatory proteins are known to control the *gadA* and *gadBC* loci (6).

TCSs contribute to the regulation of AR system 2 and render the cells acid resistant. The EvgS/EvgA TCS is positioned at the top of the system; overproduction of the EvgA response regulator, or a constitutively active EvgS (HK) mutant, leads to an AR2-dependent acid resistance phenotype in exponentially growing cells (6–9). As shown in Fig. 1, EvgS/EvgA triggers a transcriptional cascade of the regulators EvgA, YdeO, and GadE that induces the expression of AR genes such as *gadA* and *gadBC*. GadE, in particular, serves as the central regulator of the AR2 system. EvgS/EvgA further activates another TCS, the PhoQ/PhoP system, via a small connector protein, SafA (10–12). Activation of PhoQ/PhoP also contributes to AR in cells at their exponential-growth stage by increasing the cellular RpoS level via another connector, IraM (13, 14). It has also been reported that RcsB, a response regulator of the RcsC/RcsD/RcsB TCS (in this case, a three-component system), is necessary for expression of GadE-dependent promoters of the AR genes (15). Furthermore, since EvgS/EvgA cannot be activated in a Δ*rcsB* background, RcsB is essential for EvgS/EvgA activation (16). The RcsC/RcsD/RcsB system is known to be activated by various external stimulations, including low pH (17). However, RcsB need not be phosphorylated, nor are RcsC and RcsD histidine kinases necessary for RcsB to activate EvgS/EvgA (Y. Eguchi, unpublished data). Thus, our interest concentrates on what signals activate the EvgS sensor, which switches on the AR systems.

EvgS is a hybrid histidine kinase composed of a histidine kinase domain, receiver domain, and Hpt transmitter domain. The phosphate at the initial histidine residue in the histidine kinase domain is subsequently transferred to an aspartate residue in the receiver

Received 7 April 2014 Accepted 17 June 2014

Published ahead of print 23 June 2014

Address correspondence to Ryutaro Utsumi, utsumi@nara.kindai.ac.jp.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.01742-14.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JB.01742-14

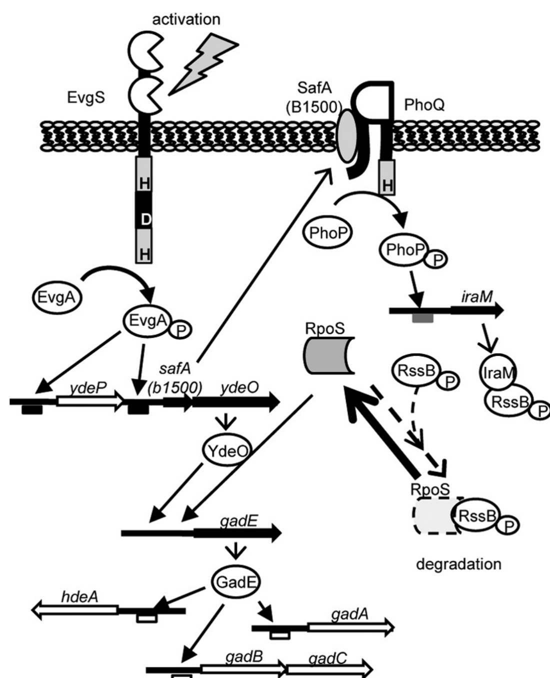

FIG 1 Activation of theEvgS sensor triggers a transcriptional cascade of the EvgA, YdeO, and GadE regulators which induces expression of AR genes (white arrows). The EvgS/EvgA system further activates the PhoQ/PhoP system, via a small protein, SafA, which increases the cellular RpoS level via IraM and RssB. Other AR genes induced by the EvgS/EvgA and PhoQ/PhoP systems and RpoS are not shown in this diagram.

domain and is relayed to the second histidine residue in the Hpt domain before being transferred to the aspartate residue in EvgA (18). Another feature of EvgS is its large periplasmic region, consisting of two tandem PBPb (bacterial periplasmic solute-binding protein) domains. These domains are also called the Venus flytrap (VFT) domains, which are ubiquitous in all kingdoms of life, and their plasticity offers several possible modes of action. Tandem periplasmic VFT domains for signal perception are found in 419 sensor kinases in 272 genomes (19). A signal peptide is predicted by SignalP 4.0 (20) at the N-terminal end of EvgS, followed by the two PBPb domains and a transmembrane region. Thus, EvgS is predicted to have a single transmembrane domain with the N-terminal end protruding into the periplasmic space. EvgS is homologous to BvgS, the major TCS regulating the virulence of *Bordetella pertussis*. Activity of BvgS, also having the two tandem VFT domains, is repressed by nicotinic acid, most likely by perception of nicotinic acid at the second VFT domain (21).

Previous reports have shown that exponential-phase cultures growing in mild acidic minimal medium (pH 5.5) induce AR by activating the EvgS/EvgA system (6, 9). However, they did not show that the EvgS sensor, and not EvgA, was actually sensing the low pH. Information concerning how EvgS is activated is also not available. In the present study, we began by confirming whether mild acidic minimal medium actually activated the EvgS sensor. A search for signals revealed that EvgS responded to high concentrations of alkali metals in addition to low pH. We have also constructed chimera sensors to determine the domains of EvgS that perceive the signals.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strains and plasmids used in this study are listed in Table S1 in the supplemental material. The strains were aerobically grown at 37°C in LB medium (1% [wt/vol] Bacto tryptone [BD Diagnostics], 0.5% [wt/vol] Bacto yeast extract [BD], 1% [wt/vol] NaCl, pH 7.5), M9 medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, and 1 g NH_4Cl per liter with the addition of 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.2% glucose, 0.0001% vitamin B_1, and 0.004% Casamino Acids [wt/vol], pH 5.5 or 7.5) (22), or minimal A medium [10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, and 0.5 g sodium citrate·$2H_2O$ per liter with the addition of 1 mM $MgSO_4$, 0.2% glucose, 0.0005% vitamin B_1, and 0.004% (wt/vol) Casamino Acids, pH 5.5 or 7.5] (22). The pH of the media was adjusted with HCl or NaOH. When necessary, selective antibiotics (100 μg/ml ampicillin, 25 μg/ml kanamycin, or 25 μg/ml chloramphenicol) were added to the medium. Mutants were obtained by a series of P1 transductions (see Table S1). Arabinose was added to the culture at a final concentration of 0.002% or 0.2% (wt/vol) for cells transformed with plasmids carrying an arabinose promoter.

Construction of reporter strains. Oligonucleotide primers used in this study are listed in Table S2 in the supplemental material. Chromosomal *ydeP-lacZ* and *emrKY-lacZ* operon fusions were constructed by the method of Ellermeier et al. (23). A chloramphenicol resistance cassette was inserted into the chromosome of strain MG1655 using the λ Red recombination method (24). FLP recombination target (FRT)-flanked chloramphenicol resistance cassettes with 5′ and 3′ ends of 35- to 40-nucleotide (nt) fragments homologous to the sequence just downstream of the stop codon of *ydeP* or *emrY* were PCR amplified by using primer pair ydeP-P2-F and ydeP-P1-R and primer pair emrY-P2-F and emrY-P1-R, with pKD3 as a template. The PCR products were electroporated into MG1655 containing the pKD46 plasmid, which carries the λ Red recombinase genes, and integrants were selected by chloramphenicol resistance. Insertion of the chloramphenicol cassette at the targeted site on the chromosome was PCR verified with primer pair ydeP-F1 and ydeP-R1 and primer pair emrY-F1 and emrY-R1. After removal of pKD46, the chloramphenicol cassette was eliminated by transforming the pCP20 plasmid, which carries the FLP recombinase gene. The pKG137 plasmid was further electroporated into the cells for the integration of the plasmid into the FRT site, and pCP20 was removed. Integration of the plasmid to the targeted chromosomal location was verified by PCR using primer pair ydeP-F1 and Lac and primer pair emrY-F1 and Lac.

Sequencing of *evgS*. Using the chromosomal DNA of strains KMY1 and MG1655 *emrKY-lacZ* as a template, two DNA fragments ($evgS_{1-1912}$ and $evgS_{1779-3594}$) were amplified by primers EvgS-F1-EcoRI and EvgS-R1-EcoRI and primers EvgS-F2-EcoRI and EvgS-R2-EcoRI. These fragments were EcoRI treated, ligated into the EcoRI site of pUC18, and sequenced.

Construction of EvgS variants. Site-directed mutagenesis at Leu152 of EvgS was performed using a QuikChange site-directed mutagenesis kit (Stratagene), primers listed in Table S2 in the supplemental material (sites of mutation are underlined), and pBADevgS plasmid. pBADevgSΔSD was constructed using evgS 531-F and evgS 29-R primers and pBADevgS as a template for PCR. The PCR product was treated with DpnI (Toyobo) to degrade the template plasmid, phosphorylated at its 5′ end with polynucleotide kinase (Toyobo), and self-ligated. All constructed plasmids were confirmed by DNA sequencing.

Construction of EvgS-PhoQ chimeras. Primers (phoQ 43-F, phoQ 190-R, EvgS SD-F, EvgS SD-R, and EvgS linker-R) were phosphorylated at the 5′ end with polynucleotide kinase. The phosphorylated primer pairs (phoQ 43-F and phoQ 190-R, EvgS SD-F and EvgS SD-R, and EvgS SD-F and EvgS linker-R) and pBADphoQ or pBADevgS as a template were used to PCR amplify the 5′-phosphorylated DNA fragments encoding $PhoQ_{43-190}$, $EvgS_{1-558}$, and $EvgS_{1-710}$. These fragments were blunt ligated to PCR-amplified DNA fragments with primer pair evgS 531-F and evgS23-R versus pBADevgS or primer pair PhoQ HisKA-F and pBAD back and primer pair PhoQ cyt-F and pBAD back versus

pBADphoQ. All constructed plasmids were confirmed by DNA sequencing.

β-Galactosidase assay. A single colony of an *E. coli* strain was inoculated in 5 ml of LB medium containing appropriate antibiotics and grown overnight with aeration at 37°C. Ten ml of minimal medium with antibiotics and arabinose for induction was inoculated with 0.1 ml of the overnight culture and grown at 37°C with aeration. When the culture reached a cell optical density at 600 nm (OD_{600}) of 0.2 to 0.3, cells were subjected to β-galactosidase assays performed in duplicate and the results were expressed as Miller units (25). The data shown are means and standard errors of the results determined with at least three individual cultures. Statistical analysis was performed by unpaired *t* tests using Prism software (GraphPad).

Acid resistance assay. Acid resistance was determined by the method of Masuda and Church (26). Cells were grown to their mid-exponential phase (OD_{600} = 0.2 to 0.3) in minimal medium, and 50 μl of the cell culture was transferred to 1.95 ml of prewarmed LB medium (adjusted to pH 2.5 with HCl) and incubated at 37°C for 1 h. Viable cells in the acidified culture were counted by plating serial dilutions onto LB plates. The percentage of acid survival was calculated as the number of viable cells remaining after acid treatment divided by the viable counts before acid treatment. Each experiment was repeated at least three times, and the means and standard errors of the results were determined.

Detection of EvgS variants and chimera sensors in the membrane fraction. Cells were grown in 10 ml of supplemented minimal medium as described for the β-galactosidase assay to OD_{600} = 0.2 to 0.3. From the cultures, 0.4 ml was sampled for the β-galactosidase assay, and the remaining 9.6-ml cultures were centrifuged for 10 min at 2,300 × *g* and 4°C. The pellets were resuspended with 500 μl of lysis buffer (100 mM NaCl, 50 mM Tris-HCl, pH 8) and sonicated for cell disruption. The cell lysates were centrifuged for 10 min at 2,300 × *g* and 4°C, and the supernatants were further centrifuged for 30 min at 17,800 × *g* and 4°C to collect the membrane fractions. The pellets were suspended in 20 μl of lysis buffer and 5 μl of 5× sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and 10 μl of each sample was subjected to SDS-PAGE. The electrophoresed proteins were then transferred to a polyvinylidene difluoride membrane (Immobilon-P transfer membranes; Millipore) and probed with anti-EvgS or anti-PhoQ antiserum. Detection was carried out with enhanced chemiluminescence (ECL) peroxidase-labeled anti-rabbit antibody (GE Healthcare Bio-Science) and Luminata Forte Western horseradish peroxidase (HRP) substrate (Millipore). Signals were acquired by the use of an ImageQuant Imager 400 system (GE Healthcare Bio-Science).

RESULTS

Alkali metals in addition to acidic pH are required for EvgS/EvgA activation. In order to determine the activating signals for the EvgS sensor, we constructed chromosomal *ydeP*-*lacZ* and *emrKY*-*lacZ* operon fusion strains for analyzing the activity of the EvgS/EvgA system. The *ydeP* and *emrK*-*emrY* genes are directly regulated by EvgA (7, 27), and their expression is induced in the presence of a constitutively active mutant of EvgS (EvgS1) (8, 27, 28).

Using the MG1655 *ydeP*-*lacZ* strain, we attempted to activate EvgS/EvgA in a minimal medium at low pH (6, 9). When minimal A medium was used, *ydeP* expression was induced at pH 5.5. However, when M9 medium was used, no activation was observed at pH 5.5 (Fig. 2A). We compared the compositions of the two minimal media and noticed the difference in Na^+ and K^+ concentrations. $[Na^+]$ and $[K^+]$ in M9 medium were 93.2 and 22.0 mM, respectively, whereas those in minimal A medium were 16.7 and 114.8 mM, respectively. The EG minimal medium used for EvgS/EvgA activation in the former study (6) had $[Na^+]$ and $[K^+]$ of 5.1 and 153.7 mM, respectively. Comparing these values, we assumed that the high concentration of K^+ may be the activating signal or that Na^+ may be the deactivating signal for EvgS/EvgA. As shown in Fig. 2B, adding KCl to M9 medium at final concentrations of 50 and 100 mM induced *ydeP* expression at pH 5.5 but not at pH 7.5. To our surprise, addition of NaCl and LiCl to M9 medium at pH 5.5 also induced *ydeP* expression. However, $MgCl_2$ did not induce *ydeP* expression, indicating that neither Mg^{2+} nor Cl^- was involved in the induction (Fig. 2B). These results strongly suggested that, in addition to low pH, high concentrations of alkali metals such as Na^+ and K^+ are required for activating the EvgS/EvgA system. Similarly to *ydeP* expression, *emrKY* expression was also induced by the addition of NaCl or KCl to M9 medium at pH 5.5 in MG1655 *emrKY*-*lacZ*, confirming that the EvgS/EvgA system was activated by these signals (Fig. 2C).

In order to confirm that this effect of a high concentration of alkali metals was not a consequence of high osmolality, sucrose, proline, and choline chloride were each added to M9 medium (pH 5.5) at final concentrations of 100 and 200 mM. Osmolalities of the media supplemented with solutes at a final concentration of 200 mM were higher than those seen with NaCl- or KCl-supplemented M9 medium (pH 5.5) at a final concentration of 100 mM (see Fig. S2B in the supplemental material), and their effect on EvgS activity was examined. As a result, activation of EvgS did not occur by the addition of sucrose and proline, eliminating the possibility that osmotic pressure was the signal (see Fig. S2A). However, a moderate increase in activity was observed in the samples with choline chloride added. This may have been caused by the high concentration of monovalent choline cation. Furthermore, EvgS quickly responds to the signals; an increase in *ydeP* expression was observed as early as 5 min after exposure to the signals (see Fig. S3). The initial surge followed by a slower increase in *ydeP* expression is considered to represent feedback repression mediated by the YdeO and PhoP transcription factors, as reported by Burton et al. (9).

The pH range for EvgS/EvgA activation was determined by growing MG1655 *ydeP*-*lacZ* in M9 medium (pH 7.5) until the exponential phase (OD_{600} = 0.1 to 0.2), after which cells were collected by centrifugation and resuspended in M9 medium at various pH levels with the addition of 100 mM KCl. Cells were cultured for 30 min at 37°C, and their β-galactosidase activity was measured. As shown in Fig. 3, EvgS/EvgA activation occurred under mildly acidic conditions of from pH 5.0 to 6.0 and not at pH 4.5, 6.5, or 7.

Activation of the EvgS/EvgA system by the signals is EvgS dependent. Induction of *ydeP* expression was not observed in an *evgS*-deleted MG1655 *ydeP*-*lacZ* strain grown in NaCl- or KCl-supplemented M9 medium at pH 5.5 (Fig. 4A), but the induction was recovered by complementation of *evgS* by an EvgS-expressing plasmid (Fig. 4B). These results show that the EvgS sensor responds to mildly acidic conditions in the presence of a high concentration of Na^+ or K^+ and is activated by these signals.

EvgS activation confers acid resistance to exponentially growing cells (8, 14). We examined the acid resistance of MG1655 and MG1655 *evgS* grown in KCl-supplemented M9 medium (pH 7.5 and 5.5). As shown in Fig. 5, acid resistance was induced in MG1655 in KCl-supplemented M9 medium only at pH 5.5, confirming that these signals activate the EvgS sensor.

L152 in the periplasmic region of EvgS is involved in EvgS activation. Strain KMY1 is another reporter strain of the EvgS/EvgA system which has been used in our laboratory (27, 28). This

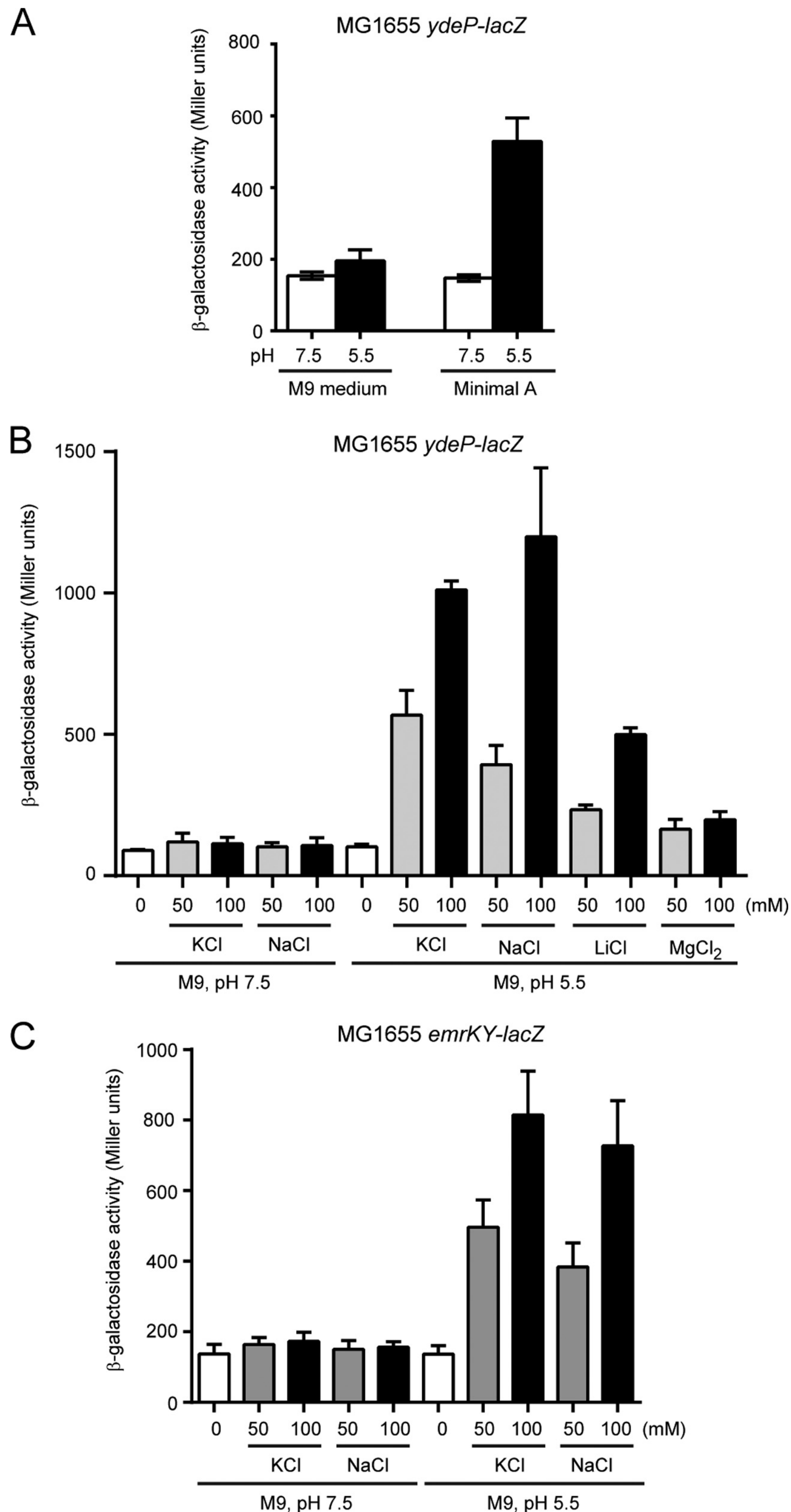

FIG 2 Alkali metals, in addition to acidic pH, are required for EvgS/EvgA activation. Transcriptional activities of *ydeP* in strain MG1655 *ydeP-lacZ* (A and B) and *emrKY* in strain MG1655 *emrKY-lacZ* (C) were measured to examine EvgS/EvgA activity. β-Galactosidase activity was measured in cultures grown to the mid-exponential phase. (A) Transcriptional activity of *ydeP* was induced only in minimal A medium adjusted to pH 5.5. (B) Addition of KCl, NaCl, and LiCl to M9 medium (pH 5.5) at final concentrations of 50 and 100 mM induced the transcriptional activity of *ydeP* but not $MgCl_2$. (C) Addition of KCl and NaCl to M9 medium (pH 5.5) at final concentrations of 50 and 100 mM induced the transcriptional activity of *emrKY*. Columns represent the means of the results of three independent experiments ± standard errors.

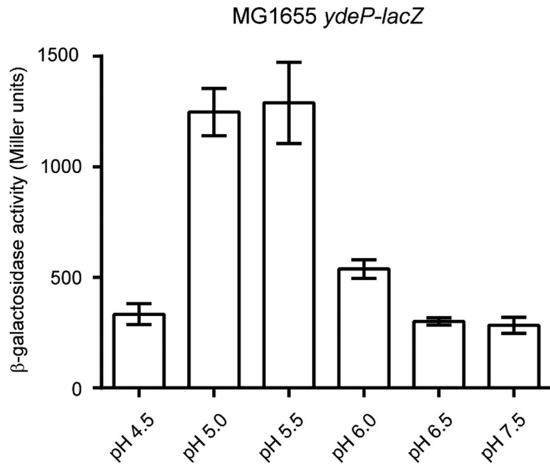

FIG 3 EvgS/EvgA is activated under mildly acidic conditions. Strain MG1655 *ydeP-lacZ* was grown in M9 medium (pH 7.5) until the exponential phase (OD_{600} = 0.1 to 0.2), and cells were collected by centrifugation and resuspended in M9 medium at various pH levels with the addition of KCl at a final concentration of 100 mM. Cells were cultured for 30 min at 37°C, and the transcriptional activity of *ydeP* was measured. Columns represent the means of the results of three independent experiments ± standard errors.

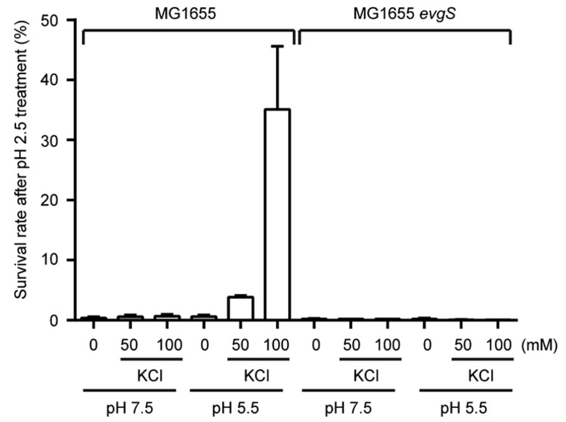

FIG 5 Acid resistance is induced by acidic pH and K^+ in an EvgS-dependent manner. Strains MG1655 and MG1655 *evgS*, which were grown to the mid-exponential phase in M9 medium (pH 7.5 and 5.5) with the addition of KCl at final concentrations of 0, 50, and 100 mM, were treated in acidic (pH 2.5) LB medium for 1 h, and their survival rates were determined. Survival rates are shown as the ratios of viable cells remaining after acid treatment. Columns represent the means of the results of three independent experiments ± standard errors.

strain is an MC4100 derivative, having a *lacZ* gene under the control of the *emrK* promoter. KMY1 was grown in KCl-supplemented M9 medium (pH 5.5), but to our surprise, no activation of the EvgS/EvgA system occurred (Fig. 6A). Thus, we compared the *evgS* sequences of the two strains, KMY1 and MG1655 *emrKY-lacZ*, and found that "CTT" encoding Leu152 in MG1655 *emrKY-lacZ* was mutated to "TTT" encoding Phe in KMY1. This residue is located in the first PBPb domain in the periplasmic region of EvgS. In order to check whether or not this mutation led to the desensitization of EvgS, an *evgS*-deleted KMY1 strain (KMY1 *evgS*) was transformed with pBADevgS (MG1655-type *evgS*) and pBADevgS L152F (KMY1-type *evgS*). When these two transformants were grown in KCl-supplemented M9 medium (pH 5.5) and assayed, EvgS activation was observed only in the pBADevgS-transformed strain (Fig. 6B). Similar expression levels of EvgS and EvgS L152 in the cell membrane were confirmed by probing with an anti-EvgS antiserum (Fig. 6B). The pBADevgS and pBADevgS L152F plasmids were also transformed into MG1655 *evgS ydeP-lacZ* and assayed in KCl-supplemented M9 medium (pH 5.5). As was the case in KMY1 *evgS*, EvgS activation was observed only by complementation with pBADevgS (Fig. 6C), confirming that L152F led to the desensitization of EvgS in strain KMY1. Furthermore, EvgS L152 in pBADevgS was changed to Ala, Arg, Glu, Ile, and Tyr and was analyzed in MG1655 *evgS ydeP-lacZ*. As shown in Fig. 6D, all the mutants were expressed in the membrane at levels comparable to those seen with the wild-type EvgS strain. EvgS L152E and L152I showed strong activation by KCl-supplemented M9 medium (pH

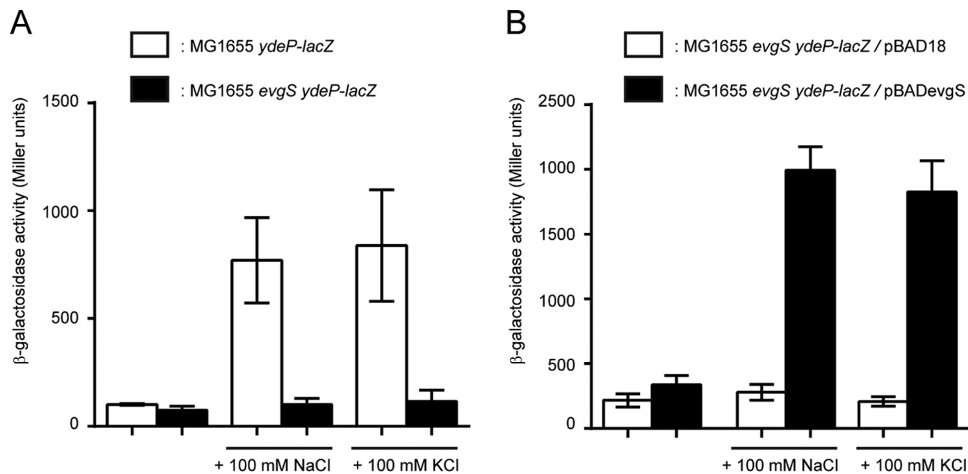

FIG 4 Activation of the EvgS/EvgA system by acidic pH and alkali metals is EvgS dependent. (A) Strain MG1655 *ydeP-lacZ* and its *evgS*-deleted mutant were grown in M9 medium (pH 5.5) with the addition of KCl or NaCl at a final concentration of 100 mM until the exponential phase, and the transcriptional activity of *ydeP* was measured. (B) Complementation of Δ*evgS* mutant. Strain MG1655 *evgS ydeP-lacZ* transformed with pBAD18 or pBADevgS was assayed as described for panel A with the addition of 0.002% arabinose for EvgS induction. Columns represent the means of the results of three independent experiments ± standard errors.

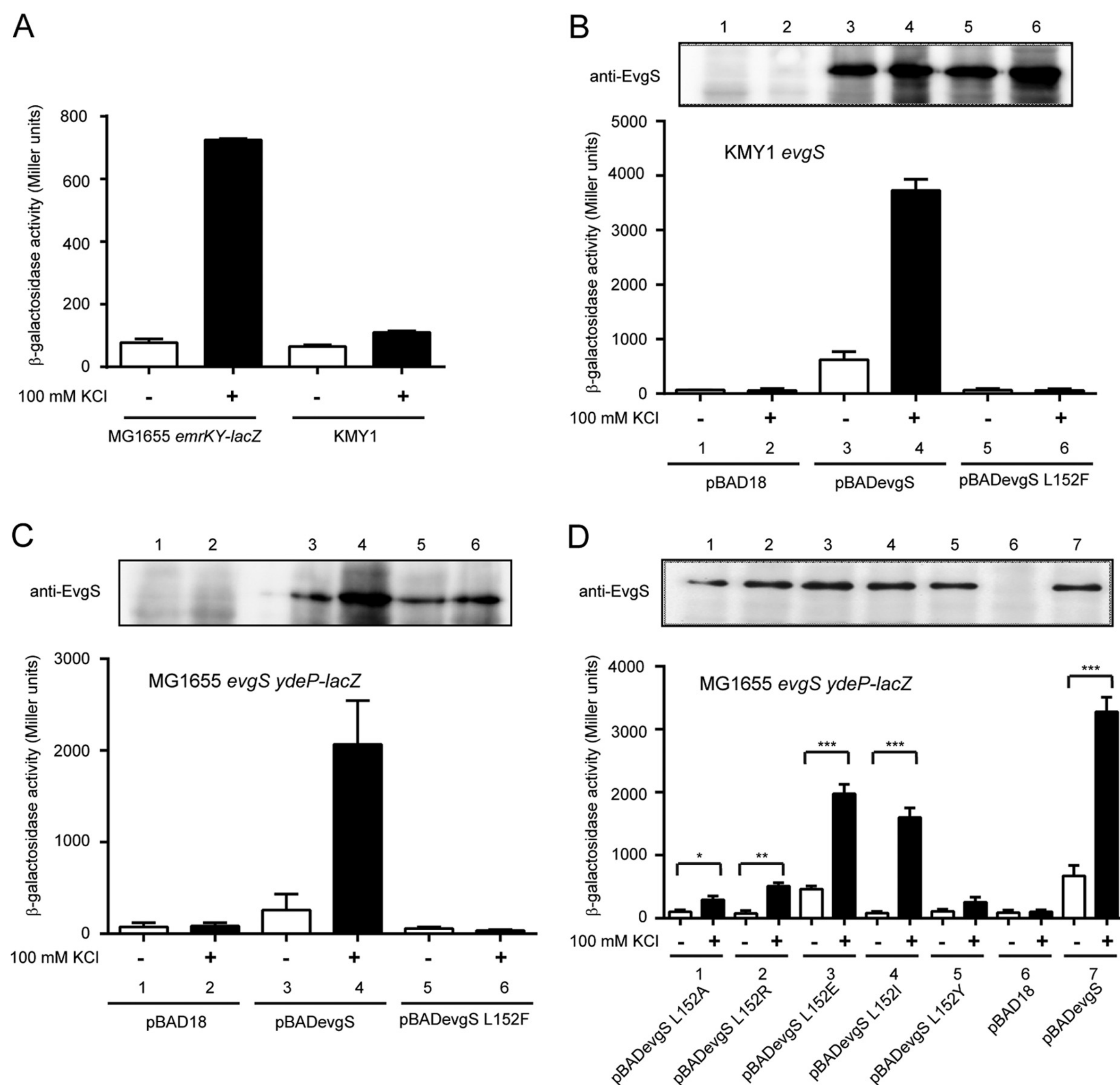

FIG 6 L152 in the periplasmic region is involved in EvgS activation. (A) Transcriptional activities of *emrKY* in strains MG1655 *emrKY-lacZ* and KMY1 were measured in M9 medium (pH 5.5) with and without the addition of KCl at a final concentration of 100 mM. (B) Transcriptional activity of *emrKY* in strain KMY1 *evgS* transformed with plasmids pBAD18, pBADevgS, and pBADevgS L152F grown in M9 medium (pH 5.5) with and without the addition of KCl at a final concentration of 100 mM and 0.002% arabinose for induction. The inset shows a Western blot using EvgS-antiserum against the cells used for the β-galactosidase assay. Numbers indicated above the lanes correspond to those shown below the columns. (C) Transcriptional activity of *ydeP* in strain MG1655 *evgS ydeP-lacZ* transformed with plasmids pBAD18, pBADevgS, and pBADevgS L152F was measured as described for panel B. The inset shows a Western blot as described for panel B. (D) Activity of EvgS L152-substituted mutants. Transcriptional activity of *ydeP* in strain MG1655 *evgS ydeP-lacZ* transformed with plasmids pBADevgS L152A, pBADevgS L152R, pBADevgS L152E, pBADevgS L152I, pBADevgS L152Y, pBAD18, and pBADevgS was measured as described for panel B. The inset shows a Western blot as described for panel B. Expression levels of EvgS and its mutants were examined only in cells grown in the presence of 100 mM KCl. Statistical significance is indicated by asterisks as follows: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$. Columns represent the means of the results of at least three independent experiments ± standard errors.

5.5), and L152A and L152R showed moderate activation, but L152Y showed no activation. The fact that L152F and L152Y mutations led to the desensitization of EvgS suggests that the aromatic ring inserted at the L152 position hindered the conformational change caused by the signals or that other factors such as changes in protein folding were responsible. EvgS L152 is quite highly conserved among *E. coli* and *Shigella* strains. When *evgS* gene sequences from 46 strains of pathogenic and nonpathogenic *E. coli* and from 8 strains of *Shigella* were examined, L152 was conserved in as many as 52 strains. The L152F mutation was found in only two *E. coli* strains, MC4100 and DH1.

The linker region is also required for recognition of acidic pH. Since L152 is located in the first PBPb domain of the periplasmic sensor domain of EvgS, we next examined whether the sensor domain is necessary for signal recognition. pBADevgSΔSD expressing EvgS without the sensor domain (EvgS lacking residues 30 to 530) and pBADevgP expressing a PhoQ-EvgS chimera (residues 43 to 190 of *E. coli* PhoQ fused between residues 1 to 23 and

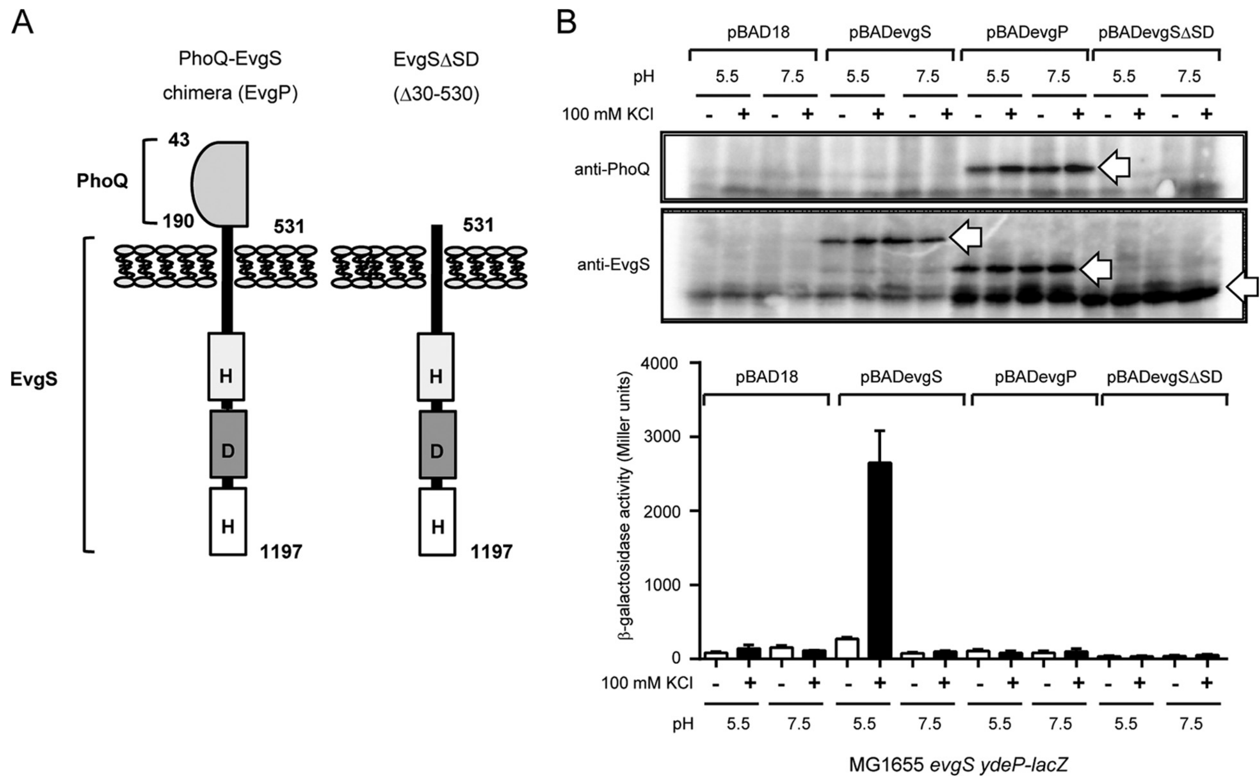

FIG 7 The periplasmic region of EvgS is necessary for activation. (A) Diagram of PhoQ-EvgS chimera (EvgP) and EvgSΔSD. Residues 43 to 190 of PhoQ were fused between residues 1 and 23 and residues 531 and 1197 of EvgS in chimera EvgP, and residues 30 to 530 in EvgSΔSD were deleted. (B) Transcriptional activities of *ydeP* in strain MG1655 *evgS ydeP-lacZ* transformed with plasmids pBAD18, pBADevgS, pBADevgP, and pBADevgSΔSD were measured in M9 medium (pH 5.5 and 7.5) with or without the addition of KCl at a final concentration of 100 mM and 0.002% arabinose for induction. The insets show Western blots using PhoQ antiserum and EvgS antiserum against the cells used for the β-galactosidase assay. White arrows indicate the EvgS, EvgP, and EvgSΔSD bands. Columns represent the means of the results of three independent experiments ± standard errors.

residues 531 to 1197 of EvgS) (Fig. 7A) were transformed into MG1655 *evgS ydeP-lacZ* and assayed. As a result, no activation was observed in KCl-supplemented M9 medium (pH 5.5) in either of the transformants (Fig. 7B), indicating that the sensor domain was essential for the activation of EvgS by the signals.

Next, the EvgS sensor domain was fused to the cytoplasmic region of PhoQ to examine whether it still responded to the signals. In chimera PvgS-A, the region from the N terminus to the transmembrane region of EvgS (residues 1 to 558 of EvgS) was fused to the cytoplasmic region of PhoQ (residues 215 to 486 of PhoQ) comprising the HAMP, HisKA, and HATPase_c domains, and in chimera PvgS-B, the region from the N terminus to the linker domain of EvgS (residues 1 to 710 of EvgS) was fused to the HisKA and HATPase_c domains of PhoQ (residues 267 to 486 of PhoQ) (Fig. 8A). Activation of these chimera sensors was examined with a reporter strain of the PhoQ/PhoP system because the HisKA and HATPase_c domains derive from PhoQ. We transformed the plasmids expressing these chimeras into the MC4100 *phoQ* $P_{mgtA\text{-}lacZ}$ strain (MG1607; *lacZ* is fused to the *mgtA* promoter which is directly regulated by PhoP) for the assay. This reporter strain derives from MC4100 and has a desensitized EvgS L152F.

Expression of PhoQ and chimera sensors was first induced with 0.002% arabinose. PhoQ and PvgS-B were detected in a Western blot of membrane samples probed with anti-PhoQ antiserum (Fig. 8B), but PvgS-A was not (data not shown). Thus, expression of PvgS-A in the pBADpvgS-A transformant was induced with 0.2% arabinose (Fig. 8C). When these transformants were assayed, PvgS-B was activated in the presence of both a high concentration of KCl and acidic pH, but PhoQ was not. This suggested that the activated conformation of the EvgS periplasmic and linker domains caused by the signals was efficiently transferred to the PhoQ HisKA and HATPase_c domains for their activation. On the other hand, PvgS-A did not respond to acidic pH but did respond to KCl, indicating that the conformational change of the periplasmic and/or transmembrane regions of EvgS was able to activate the PhoQ HisKA and HATPase_c domains via the PhoQ HAMP domain. Under our experimental conditions, the PhoQ sensor, which has been reported by Prost et al. (29) to be responsive to acidic pH, was not activated at pH 5.5 (Fig. 8B). This may have been due to the differences in the media used, namely, the modified N-minimal medium [5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 1 mM $MgCl_2$, 0.2% glucose, and 0.1% Casamino Acids, containing 100 mM MES (morpholineethanesulfonic acid) instead of 100 mM Tris] used by Prost et al. (29) and the M9 medium used in the present study. Activation by acidic pH was, however, seen in the pBADpvgS-B transformant. These results suggested that whereas K^+ can be sensed by the periplasmic sensor and/or transmembrane domains of EvgS, acidic pH requires both the linker and the periplasmic domains to be perceived by EvgS.

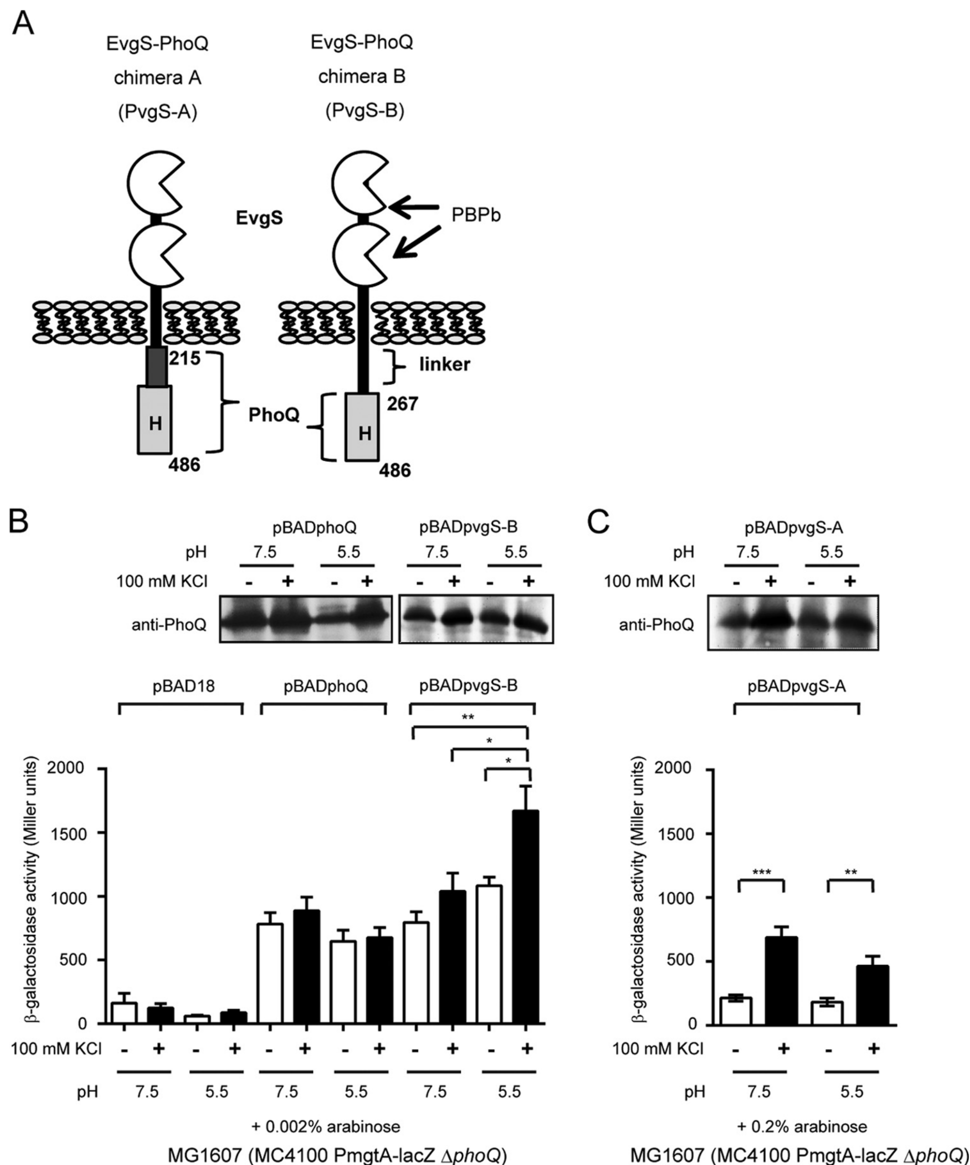

FIG 8 Signal recognition by EvgS-PhoQ chimera sensors. (A) Diagram of EvgS-PhoQ chimeras, PvgS-A, and PvgS-B. Residues 1 to 558 of EvgS were fused to residues 215 to 486 of PhoQ in chimera PvgS-A, and residues 1 to 710 of EvgS were fused to residues 267 to 486 of PhoQ in chimera PvgS-B. (B and C) Transcriptional activities of *mgtA* in strain MG1607 transformed with plasmids pBAD18, pBADphoQ, pBADpvgS-B, and pBADpvgS-A were measured in M9 medium (pH 5.5 and 7.5) with and without the addition of KCl at a final concentration of 100 mM. Cells in panel B were induced with 0.002% arabinose and those in panel C with 0.2% arabinose. The insets show Western blots using PhoQ antiserum against the cells used for the β-galactosidase assay. Columns represent the means of the results of at least three independent experiments ± standard errors. Statistical significance is indicated by asterisks as follows: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

DISCUSSION

In the present study, we found that the EvgS sensor requires the presence of a high concentration of monovalent cations such as K^+ and Na^+ for its activation by low pH. EvgS activation at various final concentrations of Na^+ and K^+ in M9 medium at pH 5.5 was assayed using MG1655 *ydeP-lacZ*. As shown in Fig. S1 in the supplemental material, EvgS was activated from $[Na^+] \geq 150$ mM and $[K^+] \geq 75$ mM. Since both cations activated EvgS and the basal $[Na^+]$ and $[K^+]$ in M9 medium are 93.2 and 22.0 mM, respectively, the effect of the sum of $[Na^+]$ and $[K^+]$ in the culture was considered. In this case, EvgS was activated from between $[Na^+]$ plus $[K^+]$ of 122 to 172 mM in the NaCl-supplemented culture and $[Na^+]$ plus $[K^+]$ of 143 to 168 mM in the KCl-supplemented culture. These results suggested that EvgS activation occurred from $[Na^+]$ plus $[K^+] \geq$ ca. 150 mM in M9 medium (pH 5.5).

Since fluctuations in pH are among the most frequently encountered environmental stresses to which bacteria are exposed, various pH sensors have been reported. In *E. coli*, CadC and PhoQ are among the sensors that respond to mildly acidic pH. CadC is a membrane-integrated transcriptional regulator which combines sensory, signal transduction, and DNA-binding activities within a single polypeptide. This one-component system is activated at below pH 6.6 in the presence of lysine. Activation of CadC induces

the *cadBA* operon, encoding the lysine decarboxylase and lysine/cadaverine transporter of the Cad system (30, 31), which is AR system 4 in *E. coli*. Lysine is not directly sensed by CadC, but low pH is sensed by the periplasmic domain of CadC (32). A cluster of negatively charged amino acids in the CadC periplasmic domain was found to be crucial for pH detection, and a model showing that protonation of the acidic amino acid side chains reduces repulsive forces between the two subdomains and/or between two monomers within a CadC dimer for activation has been proposed (33).

PhoQ is a histidine kinase of the PhoQ/PhoP TCS and detects several signals, including divalent cations, antimicrobial peptides, and low pH. These signals are also sensed at the periplasmic domain, which has been demonstrated by reconstitution of *Salmonella* PhoQ in vesicles with the signals inside (29). *E. coli* PhoQ has also been shown to be responsive to low pH in N-minimal medium (34). However, when N-minimal medium was changed to M9 medium in the present study, activation of PhoQ was not observed (Fig. 8; compare pH 7.5 to 5.5 for the pBADphoQ-transformed strain). As is the case in EvgS, PhoQ may be responding to some component(s) in N-minimal media which is not included in M9 medium in addition to acidic pH.

Analysis of the EvgS-PhoQ chimeras showed that the activated conformation of the EvgS periplasmic and linker domains can be transduced to the PhoQ histidine kinase and catalytic domains (Fig. 8). This indicates that the histidine kinase and catalytic domains of EvgS and PhoQ share a change in their conformation for activation. Furthermore, changes in $[K^+]$ were sensed at the periplasmic domain, but both the periplasmic and cytoplasmic linker domains were necessary to transduce the low pH signal to the histidine kinase domain. Low pH may be sensed at the periplasmic domain, as is the case for CadC and PhoQ, since deletion of the periplasmic domain of EvgS or swapping it to PhoQ periplasmic domain changes the sensor nonresponsive to low pH. In addition, L152, which is conserved in EvgS and necessary for responding to the signals (Fig. 6), is also in the periplasmic domain. In this case, the linker domain may be necessary only for the transduction of the periplasmic conformational change caused by the pH change. However, as activation by $[K^+]$ can be transduced to the PhoQ HAMP domain and that by low pH cannot (Fig. 8), we prefer to assume that the EvgS linker itself is related to the perception of low pH.

The chemoreceptors Tsr and Tar sense low cytoplasmic pH (pH_i) at their cytoplasmic linker region which connects the second transmembrane helix to the first methylation helix. Umemura et al. (35) have characterized various chimeras and localized the pH_i-sensing region to R259 to H267 of Tar and G261 to D269 of Tsr. The pH_i in *E. coli* is maintained at around 7.5 over a range of extracellular pH values from 5.0 to 9.0 (36). Although Umemura et al. had added weak acids to their culture medium to disrupt the strong pH_i homeostasis for obtaining pH_i change, the pH of the acidified M9 medium used in the present study was adjusted by the use of HCl. It has been reported that when HCl was added to change the external pH from 7.5 to 5.5, the cytoplasmic pH fell within 10 to 20 s to pH 5.6 to 6.5 but recovered rapidly to around pH 7 within 30 s after HCl addition, followed by slower recovery over the next 5 min to a pH close to 7.5 (37). Since we grew the cells in M9 medium adjusted to pH 5.5 by HCl, the pH_i may not have shifted to the acidic range. Thus, there is a possibility that the EvgS linker domain may perceive changes of other factors such as proton motive force, redox potential, or membrane potential caused by the proton gradient at the inner membrane.

The linker domain of EvgS is similar to the PAS domain in BvgS (38). The ArcB anaerobic sensor kinase also has a PAS domain between its transmembrane and histidine kinase domains. Under aerobic growth conditions, the ubiquinone electron carriers are proposed to silence the kinase activity of ArcB by oxidizing two PAS-located redox-active cysteine residues (C^{180} and C^{241}) that participate in intermolecular disulfide bond formation (39, 40). On the other hand, the menaquinone electron carriers are required for activation of ArcB upon a shift from aerobic to anaerobic growth conditions (41). The *in vitro* autophosphorylation activity of the cytoplasmic BvgS and EvgS (without the periplasmic and transmembrane region) kinase was also inhibited by oxidized ubiquinone and not by other electron carriers such as menaquinone, NAD, and flavin adenine dinucleotide (FAD) (38). Considering that EvgS is not activated under anaerobic conditions (Eguchi, unpublished), factors such as ubiquinone interacting against the linker domain may be involved in the pH sensing.

In conclusion, we have identified two signals (mildly acidic pH and high concentrations of alkali metals) which are required for activation of the EvgS sensor. EvgS sensed these signals through different domains, indicating the complexity of signal perception.

ACKNOWLEDGMENTS

We thank James M. Slauch for providing the pKG137 plasmid and Hiromi Ohta for the osmolality measurement. We also thank Maiko Minami, Tetsuya Morimoto, Chie Kishi, Etsu Murakami, Ayako Yamamoto, Tomoko Haneda, and Mei Yamanaka for technical assistance.

This work was supported by the Institute for Fermentation, Osaka, a Grant-in-Aid for Scientific Research (A, 20248012) from the Japan Society for the Promotion of Science (JSPS), MEXT-Supported Program for the Strategic Research Foundation at Private Universities, 2011–2015 (S1101035), and the Hayashi Memorial Foundation for Female Natural Scientists.

REFERENCES

1. **Gotoh Y, Eguchi Y, Watanabe T, Okamoto S, Doi A, Utsumi R.** 2010. Two-component signal transduction as potential drug targets in pathogenic bacteria. Curr. Opin. Microbiol. **13**:232–239. http://dx.doi.org/10.1016/j.mib.2010.01.008.
2. **Foster JW.** 2004. *Escherichia coli* acid resistance: tales of an amateur acidophile. Nat. Rev. Microbiol. **2**:898–907. http://dx.doi.org/10.1038/nrmicro1021.
3. **Smith DK, Kassam T, Singh B, Elliott JF.** 1992. *Escherichia coli* has two homologous glutamate decarboxylase genes that map to distinct loci. J. Bacteriol. **174**:5820–5826.
4. **Hersh BM, Farooq FT, Barstad DN, Blankenshorn DL, Slonczewski JL.** 1996. A glutamate-dependent acid resistance gene in *Escherichia coli*. J. Bacteriol. **178**:3978–3981.
5. **Castanie-Cornet MP, Penfound TA, Smith D, Elliot JF, Foster JW.** 1999. Control of acid resistance in *Escherichia coli*. J. Bacteriol. **181**:3525–3535.
6. **Ma Z, Masuda N, Foster JW.** 2004. Characterization of EvgAS-YdeO-GadE branched regulatory circuit governing glutamate-dependent acid resistance in *Escherichia coli*. J. Bacteriol. **186**:7378–7389. http://dx.doi.org/10.1128/JB.186.21.7378-7389.2004.
7. **Masuda N, Church GM.** 2003. Regulatory network of acid resistance genes in *Escherichia coli*. Mol. Microbiol. **48**:699–712. http://dx.doi.org/10.1046/j.1365-2958.2003.03477.x.
8. **Itou J, Eguchi Y, Utsumi R.** 2009. Molecular mechanism of transcriptional cascade initiated by the EvgS/EvgA system in *Escherichia coli* K-12. Biosci. Biotechnol. Biochem. **73**:870–878. http://dx.doi.org/10.1271/bbb.80795.
9. **Burton NA, Johnson MD, Antczak P, Robinson A, Lund PA.** 2010. Novel aspects of the acid response network of *E. coli* K-12 are revealed by

a study of transcriptional dynamics. J. Mol. Biol. **401:**726–742. http://dx.doi.org/10.1016/j.jmb.2010.06.054.

10. **Eguchi Y, Okada T, Minagawa S, Oshima T, Mori H, Yamamoto K, Ishihama A, Utsumi R.** 2004. Signal transduction cascade between EvgA/EvgS and PhoP/PhoQ two-component systems of *Escherichia coli*. J. Bacteriol. **186:**3006–3014. http://dx.doi.org/10.1128/JB.186.10.3006-3014.2004.
11. **Eguchi Y, Itou J, Yamane M, Demizu R, Yamato F, Okada A, Mori H, Kato A, Utsumi R.** 2007. B1500, a small membrane protein, connects the two-component systems EvgS/EvgA and PhoQ/PhoP in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **104:**18712–18717. http://dx.doi.org/10.1073/pnas.0705768104.
12. **Eguchi Y, Ishii E, Yamane M, Utsumi R.** 2012. The connector SafA interacts with the multi-sensing domain of PhoQ in *Escherichia coli*. Mol. Microbiol. **85:**299–313. http://dx.doi.org/10.1111/j.1365-2958.2012.08114.x.
13. **Bougdour A, Cunning C, Baptiste PJ, Elliott T, Gottesman S.** 2008. Multiple pathways for regulation of sigmaS (RpoS) stability in *Escherichia coli* via the action of multiple anti-adaptors. Mol. Microbiol. **68:**298–313. http://dx.doi.org/10.1111/j.1365-2958.2008.06146.x.
14. **Eguchi Y, Ishii E, Hata K, Utsumi R.** 2011. Regulation of acid resistance by connectors of two-component signal transduction systems in *Escherichia coli*. J. Bacteriol. **193:**1222–1228. http://dx.doi.org/10.1128/JB.01124-10.
15. **Castanié-Cornet MP, Cam K, Bastiat B, Cros A, Bordes P, Gutierrez C.** 2010. Acid stress response in *Escherichia coli*: mechanism of regulation of *gadA* transcription by RcsB and GadE. Nucleic Acids Res. **38:**3546–3554. http://dx.doi.org/10.1093/nar/gkq097.
16. **Johnson MD, Burton NA, Gutierrez B, Painter K, Lund PA.** 2011. RcsB is required for inducible acid resistance in *Escherichia coli* and acts at *gadE*-dependent and -independent promoters. J. Bacteriol. **193:**3653–3656. http://dx.doi.org/10.1128/JB.05040-11.
17. **Clarke EJ, Voigt CA.** 2011. Characterization of combinatorial patterns generated by multiple two-component sensors in *E. coli* that respond to many stimuli. Biotechnol. Bioeng. **108:**666–675. http://dx.doi.org/10.1002/bit.22966.
18. **Perraud AL, Kimmel B, Weiss V, Gross R.** 1998. Specificity of the BvgAS and EvgAS phosphorelay is mediated by the C-terminal HPt domains of the sensor proteins. Mol. Microbiol. **27:**875–887. http://dx.doi.org/10.1046/j.1365-2958.1998.00716.x.
19. **Jacob-Dubuisson F, Wintjens R, Herrou J, Dupré E, Antoine R.** BvgS of pathogenic Bordetellae: a paradigm for sensor kinases with Venus flytrap perception domains, p 57–83. *In* Gross R, Beier D (ed), Two-component systems in bacteria. Caister Academic Press, Norfolk, United Kingdom.
20. **Petersen TN, Brunak S, Heijne G, Nielsen H.** 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods **8:**785–786. http://dx.doi.org/10.1038/nmeth.1701.
21. **Herrou J, Bompard C, Wintjens R, Dupre E, Willery E, Villeret V, Locht C, Antoine R, Jacob-Dubuisson F.** 2010. Periplasmic domain of the sensor-kinase BvgS reveals a new paradigm for the Venus flytrap mechanism. Proc. Natl. Acad. Sci. U. S. A. **107:**17351–17355. http://dx.doi.org/10.1073/pnas.1006267107.
22. **Miller JH.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Plainview, NY.
23. **Ellermeier CD, Janakiraman A, Slauch JM.** 2002. Construction of targeted single copy lac fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. Gene **290:**153–161. http://dx.doi.org/10.1016/S0378-1119(02)00551-6.
24. **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. http://dx.doi.org/10.1073/pnas.120163297.
25. **Miller JH.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
26. **Masuda N, Church GM.** 2002. *Escherichia coli* gene expression responsive to levels of the response regulator EvgA. J. Bacteriol. **184:**6225–6234. http://dx.doi.org/10.1128/JB.184.22.6225-6234.2002.
27. **Kato A, Ohnishi H, Yamamoto K, Furuta E, Tanabe H, Utsumi R.** 2000. Transcription of *emrKY* is regulated by the EvgA-EvgS two-component system in *Escherichia coli* K-12. Biosci. Biotechnol. Biochem. **64:**1203–1209. http://dx.doi.org/10.1271/bbb.64.1203.
28. **Eguchi Y, Oshima T, Mori H, Aono R, Yamamoto K, Ishihama A, Utsumi R.** 2003. Transcriptional regulation of drug efflux genes by EvgAS, a two-component system in *Escherichia coli*. Microbiology **149:**2819–2828. http://dx.doi.org/10.1099/mic.0.26460-0.
29. **Prost LR, Daley ME, Le Sage V, Bader MW, Le Moual H, Klevit RE, Miller SI.** 2007. Activation of the bacterial sensor kinase PhoQ by acidic pH. Mol. Cell **26:**165–174. http://dx.doi.org/10.1016/j.molcel.2007.03.008.
30. **Watson N, Dunyak DS, Rosey EL, Slonczewski JL, Olson ER.** 1992. Identification of elements involved in transcriptional regulation of the *Escherichia coli cad* operon by external pH. J. Bacteriol. **174:**530–540.
31. **Meng SY, Bennett GN.** 1992. Regulation of the *Escherichia coli cad* operon: location of a site required for acid induction. J. Bacteriol. **174:**2670–2678.
32. **Tetsch L, Koller C, Haneburger I, Jung K.** 2008. The membrane-integrated transcriptional activator CadC of *Escherichia coli* senses lysine indirectly via the interaction with the lysine permease LysP. Mol. Microbiol. **67:**570–583. http://dx.doi.org/10.1111/j.1365-2958.2007.06070.x.
33. **Haneburger I, Eichinger A, Skerra A, Jung K.** 2011. New insights into the signaling mechanism of the pH-responsive, membrane-integrated transcriptional activator CadC of *Escherichia coli*. J. Biol. Chem. **286:**10681–10689. http://dx.doi.org/10.1074/jbc.M110.196923.
34. **Lippa AM, Goulian M.** 2009. Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. PLoS Genet. **5:**e1000788. http://dx.doi.org/10.1371/journal.pgen.1000788.
35. **Umemura T, Matsumoto Y, Ohnishi K, Homma M, Kawagishi I.** 2002. Sensing of cytoplasmic pH by bacterial chemoreceptors involves the linker region that connects the membrane-spanning and the signal-modulating helices. J. Biol. Chem. **277:**1593–1598. http://dx.doi.org/10.1074/jbc.M109930200.
36. **Slonczewski JL, Rosen BP, Alger JR, Macnab RM.** 1981. pH homeostasis in *Escherichia coli*: measurement by ^{31}P nuclear magnetic resonance of methylphosphonate and phosphate. Proc. Natl. Acad. Sci. U. S. A. **78:**6271–6275. http://dx.doi.org/10.1073/pnas.78.10.6271.
37. **Wilks JC, Slonczewski JL.** 2007. pH of the cytoplasm and periplasm of *Escherichia coli*: rapid measurement by green fluorescent protein fluorimetry. J. Bacteriol. **189:**5601–5607. http://dx.doi.org/10.1128/JB.00615-07.
38. **Bock A, Gross R.** 2002. The unorthodox histidine kinases BvgS and EvgS are responsive to the oxidation status of a quinone electron carrier. Eur. J. Biochem. **269:**3479–3484. http://dx.doi.org/10.1046/j.1432-1033.2002.03029.x.
39. **Georgellis D, Kwon O, Lin EC.** 2001. Quinones as the redox signal for the arc two-component system of bacteria. Science **292:**2314–2316. http://dx.doi.org/10.1126/science.1059361.
40. **Malpica R, Franco B, Rodriguez C, Kwon O, Georgellis D.** 2004. Identification of a quinone-sensitive redox switch in the ArcB sensor kinase. Proc. Natl. Acad. Sci. U. S. A. **101:**13318–13323. http://dx.doi.org/10.1073/pnas.0403064101.
41. **Alvarez AF, Rodriguez C, Georgellis D.** 2013. Ubiquinone and menaquinone electron carriers represent the yin and yang in the redox regulation of the ArcB sensor kinase. J. Bacteriol. **195:**3054–3061. http://dx.doi.org/10.1128/JB.00406-13.