Molecular Mechanism of Transcriptional Cascade Initiated by the EvgS/EvgA System in Escherichia coli K-12

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Using an EvgS-active mutant (evgS1) in combination with gene deletions, we clarified the molecular mechanism of the transcriptional cascade of acid resistance and multidrug resistance genes initiated by the EvgS/EvgA two-component system in Escherichia coli, followed by sequential induction of the transcriptional regulators, YdeO and GadE. Overexpression of EvgA, the response regulator of the EvgS/EvgA system, is known to induce the expression of a number of acid resistance and multidrug resistance genes, in which the EvgA-YdeO-GadE circuit is involved, but the role of the sensor EvgS in this circuit has remained unsolved. Our results suggest that the transcription cascade initiated by the EvgS/EvgA system in fact functions for acid and drug resistance in E. coli.

Key words: transcriptional cascade; EvgS/EvgA; YdeO; GadE; acid resistance

The EvgS/EvgA two-component signal transduction system in Escherichia coli (E. coli) is highly homologous to the virulence-related BvgS/BvgA system of Bordetella pertussis and the KvgS/KvgA system, which is present only in virulent strains of Klebsiella pneumoniae. A BLAST search also indicated the presence of homologous systems in other microorganisms such as Shigella flexneri, Pseudomonas aeruginosa, and Yersinia pestis. EvgS is a histidine kinase hybrid sensor composed of an N-terminal periplasmic region and a C-terminal cytoplasmic region divided into four domains: linker, transmitter, receiver, and output (Hpt). The signal recognized by EvgS at the sensory domain is transduced into a transcriptional regulation network via a cascade of phosphorylation. Autophosphorylation of the cytoplasmic region of EvgS (without the transmembrane and periplasmic regions) has been reported to be inhibited in vitro by an oxidized ubiquinone-0, as is also the case with the aerobic sensor ArcB. However, the inhibition of EvgS autophosphorylation by ubiquinone has not been confirmed in vivo experiments, and the environmental signals sensed by EvgS also remain to be determined.

By using a constitutively active mutant (evgS1 mutant), which produces a constitutively active EvgS with an F577S mutation within the linker region (also a predicted PAS, Per-Arnt-Sim, domain), Kato et al. found that the transcription of emrKY, a multi-component drug efflux pump of the major facilitator superfamliy (MFS), is positively regulated by the EvgS/EvgA system. This was followed by Eguchi et al., who examined transcriptional levels of multidrug efflux genes in an E. coli mutant expressing the constitutive mutant EvgS1 using an E. coli DNA microarray. Most of the putative multidrug efflux-related genes were below the detection level under the steady-state of the exponential growth phase, but at least five genes or operons, emrKY, mdfEF (formerly yhiUV), acrAB, and tolC, showed increased expression in the presence of the constitutive mutant EvgS1. This was supported by reports of overexpression of the regulator EvgA conferring multidrug resistance to a drug-hypersusceptible E. coli strain, which lacked the major multidrug efflux pump gene, acrB.

EvgA overexpression also conferred acid resistance to exponentially growing cells. This acid resistance was eliminated by the deletion of ydeO, or gadE, genes induced by EvgA overexpression. Microarray analysis identified two classes of operons or genes. The first class contains seven operons or genes induced by EvgA overexpression in the absence of ydeO, encoding an AraC-like regulator. The second class contains 12 operons or genes induced by YdeO overexpression. Operons or genes in the second class were induced by EvgA overexpression only in the presence of ydeO, which suggests the following model of the regulatory network of the acid resistance genes: EvgA directly upregulates operons or genes containing ydeO in the first class and indirectly upregulates operons or genes in the second class containing gadE, a LuxR-family regulator gene, via YdeO. In another study, Ma et al. used an acidified minimal medium to induce an EvgA-YdeO-GadE circuit without overexpressing EvgA. This circuit activates the glutamate-dependent acid resistance system in exponential-phase cells and is largely dependent on EvgA, but the role of the sensor EvgS in this circuit remains unsolved.

On the other hand, the transcriptome of an E. coli K-12 evgAS mutant has been compared with that of the wild type, using DNA microarray analyses. In addition, attempts to identify the evgAS mutant phenotype have included high-throughput phenotype micro-
array analyses,\textsuperscript{15)} in which nearly 2,000 different phenotypes were examined. However, neither study showed that there was a significant difference between the evgS\textsuperscript{1} mutant and the wild-type strain. Additionally, an \textit{in vitro} examination revealed low levels of EvgS autophosphorylation and EvgA transphosphorylation.\textsuperscript{16)} The results of these studies imply that the EvgS/EvgA two-component system does not function in \textit{E. coli} under the experimental conditions used and that the input signal for EvgS activation has not been found.

In the study reported in this paper, using an EvgS-active (evgS\textsuperscript{1}) mutant, we clarified the molecular mechanism of the transcriptional cascade of acid-resistance and multidrug-resistance genes initiated by the EvgS/EvgA system, followed by sequential induction of YdeO and GadE. These results suggest that the transcriptional cascade initiated by the EvgS/EvgA system in fact functions for acid and drug resistances in \textit{E. coli}.

Materials and Methods

Bacterial strains and growth conditions. The \textit{E. coli} strains and plasmids used in this study are listed in Table 1. Strains were aerobically grown at 37°C in LB medium (1% w/v Bacto tryptone (BD, Sparks, MD), 0.5% w/v Bacto yeast extract (BD), and 1% w/v NaCl, pH 7.5). When necessary, selective antibiotics were added to the medium as follows: 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, 12.5 μg/ml tetracycline, or 25 μg/ml kanamycin. Mutants were obtained by a series of P\textsubscript{i} transductions as briefly described in Table 1. \textit{C. Undigested RNA-probe DNA was P-end-labelled probe was digested for 10 min at 37°C C and then gradually cooled to 37°C and incubated 10 min at 75°C, and then gradually cooled to 37°C and incubated overnight for hybridization, followed by S1 nuclease (Takara) digestion for 10 min at 37°C. Unligated RNA-probe DNA was extracted with phenol, precipitated with ethanol, and subjected to electrophoresis on a 6% (w/v) polyacrylamide sequencing gel containing 8 M urea. The radioactivity was measured with a BAS1000 Mac (Fuji Film).}

RNA preparation and \textit{S1} nuclease analysis. Total RNA was extracted with hot phenol from a mid-exponential-phase culture (OD\textsubscript{600} 0.7–0.9) at 60°C.\textsuperscript{17)} S1 nuclease analysis was carried out as described by Kato \textit{et al.}\textsuperscript{7) In brief, a \textsuperscript{32}P-end-labelled probe was prepared by PCR using the primers listed in Table 2. MC4100 genomic DNA was a template, and ExTaq DNA polymerase (Takara, Tokyo). A mixture of \textsuperscript{32}P-end-labelled probe and 100 μg total RNA was incubated for 10 min at 75°C and then gradually cooled to 37°C and incubated overnight for hybridization, followed by S1 nuclease (Takara) digestion for 10 min at 37°C. Unligated RNA-probe DNA was extracted with phenol, precipitated with ethanol, and subjected to electrophoresis on a 6% (w/v) polyacrylamide sequencing gel containing 8 M urea. The radioactivity was measured with a BAS1000 Mac (Fuji Film, Tokyo) and analyzed with a MacBAS2.2 (Fuji Film).
Purification of EvgA and GadE. His-tagged EvgA was overexpressed in M15[pREP4] containing pEvgA, and was purified as previously described.10 MBP-GadE fusion protein was overexpressed and purified as described elsewhere,10 with modifications. In brief, pMALc2E-gadE, constructed by cloning the gadE gene into pMALc2E, was transformed into strain TB1. Cells containing pMALc2E-gadE were grown in 200 ml of LB with glucose (0.2%) and ampicillin at 30 °C with aeration, and MBP-GadE was induced by the addition of 0.5 mM IPTG. MBP-GadE was bound to amylose resin (New England Biolabs, Ipswich, MA), and eluted with 1 M maltose, followed by dialysis against storage buffer (100 mM Tris–HCl, pH 7.6, 10 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 200 mM KCl, 50% v/v glycerol) for desalting and storage. The protein concentration was determined by Bradford method (Protein Assay Kit, Bio-Rad, Hercules, CA).

DNase I footprinting analysis. The 32P-labeled probes used in the S1 analysis were also used for footprinting analysis. The probes were incubated at 37 °C for 10 min with His-tagged EvgA or MBP-GadE in 25 μl of binding buffer (50 mM Tris–HCl pH 7.8, 50 mM NaCl, 3 mM magnesium acetate, 5 mM CaCl2, 0.1 mM EDTA, 0.1 mM dithiothreitol, 250 μg/ml BSA), followed by digestion with 5 ng of DNasel (Takara) at 25 °C for 30 s. The reaction was terminated by the addition of 45 μl of DNasel stop solution (20 mM EDTA, 200 mM NaCl, 1% v/v SDS, 250 μg/ml yeast tRNA). The digested probes were precipitated by ethanol, extracted by phenol, and analyzed by electrophoresis on a 6% (v/w) polyacrylamide gel containing 8 M urea. The radioactivity was measured as described for S1 nuclease analysis.

Acid resistance assay. Acid resistance was determined by the method of Masuda and Church.11 Briefly, a single colony of an E. coli strain was inoculated in 5 ml of LB (containing ampicillin when necessary) and grown overnight with aeration at 37 °C. LB (10 ml, containing ampicillin when necessary) was inoculated with 0.1 ml of the culture for desalting and storage. The protein concentration was determined by Bradford method (Protein Assay Kit, Bio-Rad, Hercules, CA).

Results

Up-regulated genes by EvgS/EvgA system activation

In our previous DNA microarray analysis10 performed against a constitutively active mutant of histidine kinase sensor EvgS (evgS1 mutant, http://www.nara.kindai.ac.jp/nogei/seiken/array2.html), activation of the EvgS/EvgA system increased the expression of as many as 225 open reading frames (ORFs) including ones encoding transcriptional factors such as YdeO and GadE. Among them, we selected ORFs that are directly regulated by EvgA (yeoD, yeoE, yflx, yecR, and emrK)12 and those that are presumed to be regulated by YdeO and GadE (gadE, gadA, gadB, hdeA, and hdeD)12 for precise estimation of the transcriptional activation of these genes in the presence of the constitutive evgS1 mutation. A low-copy number plasmid (pOH2001D52A) carrying evgS7 and evgA with Aα substituted for Asp2 (the phosphorylation site of EvgA) was transformed into strains MG1655 and MG1655 evgA. The EvgS/EvgA system is activated in strain MG1655/pOH2001D52A, whereas it is inactive in MG1655 evgA/pOH2001D52A because EvgA in this strain cannot be phosphorylated by the activated EvgS. Total RNA was prepared from the mid-exponential phase of these cells for S1 nuclease analyses. As shown in Fig. 1A and B, the 10 genes showed increased expression after EvgS activation (lanes 1 and 2). When evgA was deleted from the constitutive evgS1 strain, the increase in expression of the 10 genes also disappeared (lanes 3 and 4), indicating that the expression of these genes is induced by EvgS activation in an EvgA-dependent manner. The gene emrK is also EvgA-dependently induced by EvgS activation.12,13

Two major transcripts of ydeO were found at positions P1 (35 nt upstream of the start codon of ydeO) and P2 (108 nt upstream of the start codon of ydeO, inside the safA ORF). However, neither 10 nor 35 boxes were found for P1 and P2, suggesting that these may not be the actual initiation sites for transcription. We obtained a faint transcript starting from 69 nt upstream of the initiation codon of safA (data not shown). The safA-ydeO operon might have been first transcribed from the transcription start site upstream of safA, followed by rapid processing of the transcript at the P1 and P2 sites. Consistent with our results, Ma et al.13 have found two transcripts by northern blot analysis of the EvgA-dependent ydeO expression: one corresponding to ydeO (presumably the P1 and P2 transcripts), and the other weaker band corresponding to safA (b1500)-ydeO.

The transcription initiation sites of the major transcripts affected by the EvgS/EvgA system activation are summarized in Table 3.

EvgS/EvgA up-regulated genes targeted by EvgA, YdeO, and GadE

Next, we constructed an evgS1 and ydeO-disrupted strain (MG2001 ydeO) and an evgS1 and gadE-disrupted strain (MG2001 gadE) in order to classify the genes regulated by YdeO or GadE. Total RNA was prepared from mid-exponential phase cells and subjected to S1 nuclease analyses against the aforementioned 10 genes. Similar transcription levels of the 5 genes directly regulated by EvgA (yeoD, yeoE, yflx, frc, and yegR) were detected among the evgS1, evgS1 ydeO, and evgS1 gadE strains (Fig. 2A, lanes 4, 5, 6) except for ydeO, which of course did not show any transcripts in the evgS1 ydeO strain. These 5 genes all possess the 18 bp consensus sequence for EvgA binding within their promoter regions,12 and we confirmed by DNase I footprinting analyses that EvgA actually bound to these consensus sequences (Fig. 3 and ref. 18 for safA-ydeO). The 18 bp consensus sequence is also present in the promoter region of emrK, and EvgA binding has been confirmed by previous DNase I footprinting analysis.12

EvgA bound to region 43 nt upstream of the transcriptional start site of safA.18 We also performed footprinting analyses for the upstream region of ydeO, but no footprint was obtained (data not shown), indicating that EvgA bound only to the upstream region of safA. The 18 bp consensus sequence was also found only in the upstream region of safA, suggesting that PsafA is the transcriptional start site of the safA-ydeO operon (Fig. 4). As for yflx, two footprints were found within the promoter region. The upstream footprint corresponded to the formerly reported 18 bp consensus sequence.12 The second footprint covered a broader range and contained a sequence similar to the 18 bp consensus sequence (Fig. 4). Although 10 boxes were
Fig. 1. Up-Regulated Genes by EvgS/EvgA System Activation.

S1 nuclease analyses were performed using total RNA from a wild strain (MG1655/pMW119, lane 1), an \textit{evgS1} strain (MG1655/pOH2001D52A, lane 2), an \textit{evgA}-deficient strain (MG1655 \textit{evgA}/pMW119, lane 3), and an \textit{evgS1 evgA}-deficient strain (MG1655 \textit{evgA}/pOH2001D52A, lane 4). Lane A+G represents the Maxam-Gilbert sequencing ladder. Asterisks show transcriptional start sites. P1 and P2 of \textit{ydeO} may not be transcriptional start sites. The arrow indicates a minor transcript of \textit{gadE}. A, Genes directly regulated by EvgA. B, Genes regulated by YdeO or GadE.
Fig. 2. Transcriptional Changes of EvgS/EvgA Up-Regulated Genes by Deletion of ydeO and gadE.
S1 nuclease analyses were performed using total RNA from a wild strain (MG1655, lane 1), a ydeO-deficient strain (MG1655 ydeO, lane 2), a gadE-deficient strain (MG1655 gadE, lane 3), an evgS1 mutant (MGR2001, lane 4), an evgS1 ydeO strain (MGR2001 ydeO, lane 5), and an evgS1 gadE (MGR2001 gadE, lane 6). A, EvgA dependent and YdeO, GadE independent expression. B, EvgA dependent and YdeO partly dependent expression. C, EvgA and GadE dependent expression.

Fig. 3. DNaseI Footprinting Analysis against EvgA.
Labeled probes of the promoter regions were incubated with 2 mM (lane 1), 4 mM (lane 2), 8 mM (lane 3), and 0 mM (lane 4) of His-tagged EvgA. Lane A+G represents the Maxam-Gilbert sequencing ladder. The protected sites are indicated by open bars. Closed bars indicate the 18 bp consensus sequence for EvgA binding. Arrows indicate transcriptional start sites.
found in all six promoters, −35 boxes were not found for safA, yegR, or emrK.

The five other genes (Fig. 2B, C) were presumed to be regulated by YdeO or GadE because these genes were all up-regulated by overexpression of YdeO in the absence of evgA.12) The gene gadE also showed a decreased level of transcripts in the evgS1 ydeO strain (Fig. 2B lane 5), suggesting its regulation by YdeO. However, a small amount of gadE transcript could still be detected in the evgS1 ydeO strain, so a regulation pathway besides that via YdeO is suspected. As for gadA, gadB, hdeA, and hdeD, no transcripts were detected in the evgS1 gadE strain (Fig. 2C lane 6), indicating that their expression was GadE dependent. We have also confirmed by DNase I footprinting analysis that GadE directly bound to the GAD boxes19,20) in the promoter regions of these genes (Figs. 5 and 6). A closer examination of the promoter sequences revealed a direct repeat of ATTTT separated by three nucleotides inside the GAD boxes.

Acid resistance conferred by EvgS/EvgA system activation

Activation of the EvgS/EvgA system confers multidrug resistance to a drug-hypersusceptible strain that lacks constitutive multidrug efflux genes accrAB, mainly by induction of mdtEF.8–10) To determine whether EvgS/EvgA system activation confers acid resistance to cells, the acid resistance of the evgS1 mutants was assayed. Exponential phase cultures of strains MG1655 (wild), MGR1655 (fadL), and MGR2001 (evgS1 mutant, fadL) were exposed to LB (pH 2.5) for 1 h. As shown in Fig. 7, MGR2001 showed a higher survival rate than MG1655 or MGR1655, indicating that EvgS/EvgA system activation did confer acid resistance.

Discussion

In the present study, we newly determined the transcription start sites of ydeP, safA, yfdX, frc, and yegR (Table 3). The ydeP gene encodes a predicted oxidoreductase and contributes to acid resistance conferred by the overproduction of EvgA.11) The small ORF, safA, encodes a small membrane protein 65 amino acids in length.18) This protein activates the sensor PhoQ of the PhoQ/PhoP two-component system, thus connecting the EvgS/EvgA and PhoQ/PhoP systems. Interestingly, PhoQ/PhoP system activation also contributes to the acid resistance conferred by EvgS/EvgA system activation (data not shown), adding another pathway to the complicated acid resistance system. The frc gene encodes a formyl-CoA transferase monomer, and yegR encodes a probable lipoprotein of unknown function. The function of the product of yfdX is also not known.

The transcription initiation sites for gadA, gadB, hdeA, and hdeD were in agreement with earlier reports.21–23) As for gadE, two transcription start sites

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**Fig. 4.** Promoter Analysis of EvgA-Activated Genes.

The bold capital letters are transcriptional start sites identified from S1 nuclease analyses (see text for details). The 18 bp consensus sequences for EvgA binding are boxed. The second footprint found in the region of yfdX is underlined, and the second consensus sequence is boxed with dashed lines. No consensus sequence for EvgA binding was found in the promoter region of gadE.
have been reported,23) at 92 and 125 nt upstream from
the start codon. Another study indicated that the tran-
scription of gadE started 21 bp upstream of the start
codon.13) In our study (Fig. 1B), we detected the start
site of the major transcript of gadE at 304 nt upstream of
the start codon. Another start site of a very minor
transcript of gadE was found at 125 nt upstream
(Fig. 1B, arrow), which corresponded to one of the
two reported sites.23) No transcripts were detected at the
21 nt or 92 nt sites in our S1 nuclease analysis. The
reason for the discrepancy in locations may be the
difference in gadE induction (we activated the EvgA/
hns system, whereas hns was deleted in the study
by Hommais et al.23) and the sensor EvgS may not have been involved in the study by Ma et al.13)).
Another reason may be that Hommais et al.23) used a
plasmid carrying the gadE gene for their primer
extension analysis, and the inserted region in their
plasmid may not have contained our major transcription
start site.

Between the evgS1 and evgS1 ydeO strains, similar
levels of transcript of gadA, gadB, hdeA, and hdeD were
observed (Fig. 2C). Thus, the small amount of gadE
transcript detected in the evgS1 ydeO strain may have
been sufficient for the induction of gadA, gadB, hdeA,
and hdeD. Ma et al.13) have reported that under
physiological conditions (log phase, minimal EG
medium, pH 5.5), much of the EvgA effect on GAD
production occurred via a route other than through EvgA
control over ydeO. They have shown by gel shift assay
that EvgA bound to the promoter region of gadE, although the consensus sequences for EvgA binding
were not found. Our present results (Figs. 2B, C, and 8)
support their model of EvgA regulating gadE without
YdeO.
tigation is required to determine whether EvgA directly regulates \textit{gadE}. The binding of YdeO to the promoter region of \textit{gadE} confirmed by gel shift assay has also been reported, but the binding sites of YdeO have not been specified, nor has its regulon other than \textit{gadE-mdtEF} been determined. Further studies are necessary to clarify how EvgA and YdeO regulate the \textit{gadE-mdtEF} operon.

To summarize, the transcriptional cascade initiated by the EvgS/EvgA system revealed in this study is illustrated in Fig. 8. The activation of the sensor EvgS phosphorylates its cognate EvgA and directly up-regulates at least six genes/operons. YdeO, one of the genes directly induced by phosphorylated EvgA, then up-regulates \textit{gadE}, the master regulator of acid resistance genes. The gene \textit{gadE} also forms an operon with \textit{mdtEF}, which encodes a multidrug efflux pump, conferring multidrug resistance. Besides the transcriptional cascade, EvgS/EvgA system activation also activates the PhoQ/PhoP system via the small membrane protein SafA. This pathway also contributes to acid resistance. It has been found that acid resistance is a critical system for the survival of \textit{E. coli}, and that various factors are involved in this system. The present study has solved part of the intricate transcriptional cascade of acid resistance genes initiated by EvgS/EvgA system activation, as one of the mechanisms of \textit{E. coli} acid resistance.

Acknowledgments

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**Table 3.** Transcription Start Sites of Major Transcripts Affected by the EvgS/EvgA Activation Determined from S1 Nuclease Analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location from the initiation codon</th>
</tr>
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<tbody>
<tr>
<td>ydeP</td>
<td>-178</td>
</tr>
<tr>
<td>safA</td>
<td>-69</td>
</tr>
<tr>
<td>yfdX</td>
<td>-31</td>
</tr>
<tr>
<td>yegR</td>
<td>-183</td>
</tr>
<tr>
<td>gadE*</td>
<td>-43</td>
</tr>
<tr>
<td>gadA*</td>
<td>-304</td>
</tr>
<tr>
<td>gadB*</td>
<td>-27</td>
</tr>
<tr>
<td>hdeA*</td>
<td>-29</td>
</tr>
<tr>
<td>hdeD*</td>
<td>-52</td>
</tr>
<tr>
<td></td>
<td>-35</td>
</tr>
</tbody>
</table>

*Corresponds to the reported transcription start site.
†Different from the reported sites.

References