Molecular Microbiology (2003) **48**(3), 699–712

Regulatory network of acid resistance genes in *Escherichia coli*

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Summary

Overexpression of the response regulator EvgA confers an acid-resistant phenotype to exponentially growing *Escherichia coli*. This acid resistance is partially abolished by deletion of *ydeP*, *yhiE* or *ydeO*, genes induced by EvgA overexpression. Microarray analysis identified two classes of operons (genes). The first class contains seven operons induced by EvgA overexpression in the absence of *ydeO*, an AraC/XylS regulator gene. The second class contains 12 operons induced by YdeO overexpression. Operons in the second class were induced by EvgA overexpression only in the presence of *ydeO*. EvgA is likely to directly upregulate operons in the first class, and indirectly upregulate operons in the second class via YdeO. Analysis using the motif-finding program ALIGNACE identified an 18 bp inverted repeat motif in six upstream regions of all seven operons directly regulated by EvgA. Gel mobility shift assays showed the specific binding of EvgA to the six sequences. Introduction of mutations into the inverted repeats upstream of *ydeP* and *b1500-ydeO* resulted in reduction in EvgA-induced *ydeP* and *ydeO* expression and acid resistance. These results suggest that EvgA binds to the inverted repeats and upregulates the downstream genes. Overexpression of YdeP, YdeO and YhiE conferred acid resistance to exponentially growing cells, whereas GadX overexpression did not. Microarray analysis also identified several GadX-activated genes. Several genes induced by overexpression of YdeO and GadX overlapped; however, *yhiE* was induced only by YdeO. The acid resistance induced by YdeO overexpression was abolished by deletion of *yhiE*, *gadC*, *slp-yhiF*, *hdeA* or *hdeD*, genes induced by YdeO overexpression, suggesting that several genes orchestrate YdeO-induced acid resistance. We propose a model of the regulatory network of the acid resistance genes.

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Introduction

The ability to survive under acidic conditions is essential for successful colonization of the mammalian host by enteric bacteria (Gordon and Small, 1993; Lin *et al*., 1995; Bearson *et al*., 1997). These organisms are faced with an extremely acidic environment (pH < 3) during their passage through the stomach and must overcome the detrimental effect of volatile fatty acids while living in the intestine. They have evolved a number of strategies enabling them to resist acidic stress.

Escherichia coli possesses glutamate and arginine decarboxylase systems as part of its acid resistance mechanism (Lin *et al*., 1996; Castanie-Cornet *et al*., 1999; De Biase *et al*., 1999). The glutamate decarboxylase system consists of three genes. Two of these genes, *gadA* and *gadB*, encode isozymes of glutamate decarboxylase, which catalyses the conversion of glutamate to γ-aminobutyrate (Smith *et al*., 1992). The third gene, *gadC*, is located downstream of *gadB* and is predicted to encode a glutamate:γ-aminobutyrate antiporter (Hersh *et al*., 1996). It has been proposed that the GadA and GadB decarboxylases and the GadC antiporter function together to help to maintain a near-neutral intracellular pH when cells are exposed to extremely acidic conditions (Small and Waterman, 1998). The arginine decarboxylase system consists of a decarboxylase encoded by *adiA* (Stim and Bennett, 1993) and an unknown arginine antiporter. *E. coli* also possesses a protein, HdeA, which has been proposed to have a chaperone-like function preventing the aggregation of periplasmic proteins denatured under extremely acidic conditions (Gajiwala and Burley, 2000). The gene *hdeB*, located downstream of *hdeA*, is predicted to code for a structural homologue of HdeA and to form heterodimers with HdeA (Gajiwala and Burley, 2000).

Co-ordinated regulation of gene expression in response to environmental stimuli is an important requirement for adaptation of bacteria to the various environments. One of the major mechanisms of signal transduction leading to specific gene expression in bacteria is the two-component system, which consists of a sensor kinase and its cognate response regulator (Hoch, 2000; West and Stock, 2001). The response regulator EvgA and sensor kinase EvgS in

E. coli are highly similar to BvgA and BvgS, respectively, which control the expression of adhesins, toxins and other virulence factors in *Bordetella pertussis* (Arico *et al*., 1991; Stibitz and Yang, 1991; Utsumi *et al*., 1994). Nishino and Yamaguchi (2001) reported that overexpression of EvgA confers multidrug resistance to a drug-hypersusceptible strain, which lacks constitutive multidrug efflux pump genes *acrAB* (Ma *et al*., 1995). In a previous study (Masuda and Church, 2002), we found that EvgA overexpression induces 37 genes, including *gadABC* and *hdeAB*, and confers acid resistance to exponentially growing cells. This acid resistance is partly abolished by deletion of *ydeP*, *ydeO* or *yhiE*, which were induced by EvgA overexpression, suggesting that the three genes are related to acid resistance. Taken together, EvgA regulates at least eight genes related to acid resistance, although the regulatory mechanism of these genes remains unclear.

In this study, we elucidate the regulatory network of acid resistance genes, including *evgA*, *ydeP*, *ydeO*, *yhiE*, *gadABC* and *hdeAB*, by a combination of microarray analysis and gene replacement techniques. We have also identified the EvgA-binding sequences using a combination of computational and genetic techniques. We propose a model regulatory network for the genes that work together to accomplish acid resistance. (Gene names in this paper are taken from http://bmb.med.miami.edu/EcoGene/EcoWeb/.)

Results and discussion

Induction of acid resistance by overexpression of YdeP, YdeO and YhiE in exponentially growing cells

We have reported that overexpression of the response regulator EvgA confers acid resistance to exponentially growing *E. coli* (Masuda and Church, 2002). This acid resistance is partly abolished by deletion of *ydeP*, *ydeO* or *yhiE*, genes induced by EvgA overexpression. YdeP is a putative oxidoreductase that has homology to the α subunit of *E. coli* formate dehydrogenase H. YdeO and YhiE are members of the AraC/XylS family (Gallegos *et al*., 1997) and LuxR superfamily (Fuqua *et al*., 1994; Robison and Church, 1994) of transcriptional regulators respectively. To confirm the involvement of YdeP, YdeO and YhiE in acid resistance, we constructed expression plasmids, introduced them into MG1655 (ATCC 47076) and tested the effect of overexpressing these proteins on acid resistance. Exponential phase cultures of each strain grown in LB broth (pH 7.0) containing IPTG were incubated in acidic LB broth (pH 2.5) for 1 h, and percentage survival values were determined as described in *Experimental procedures*. Overexpression of YdeP, YdeO and YhiE conferred acid resistance to exponentially growing cells although the degree of acid resistance varied (Fig. 1, bars 1–4). These results suggest that these genes are involved in acid resistance. Recently, Tucker *et al*. (2002) reported that 28 genes, including *slp*, *yhiF*, *hdeAB*, *hdeD*, *yhiE*, *gadA* and *gadB*, which are induced by EvgA overexpression (Masuda and Church, 2002), were induced at pH 5.5 or 4.5. They also reported that acid resistance induced by culture at pH 5.5 was abolished by *yhiE* deletion and that YhiE overexpression conferred acid resistance in minimal medium.

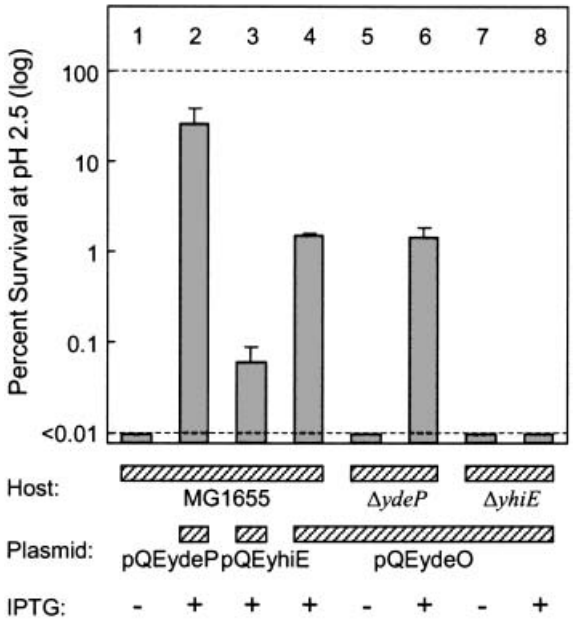

Fig. 1. Acid resistance of the YdeP, YhiE and YdeO overexpression strains. MG1655 (lanes 2–4), Δ*ydeP* (lanes 5 and 6) and Δ*yhiE* (lanes 7 and 8) harbouring pQEydeP (lane 2), pQEyhiE (lane 3) and pQEydeO (lanes 4–8) and MG1655 (lane 1) were grown to mid-log phase in LB broth (pH 7.0) with (+) or without (–) 1 mM IPTG. Cells were diluted 40-fold into LB broth (pH 2.5) and incubated for 1 h at 37°C. Initial cell densities ranged from 9.3×10^5 to 9.5×10^6 cfu ml^{-1}. Error bars represent standard errors of the mean.

Because YdeO is a member of the AraC/XylS family of transcriptional regulators (Gallegos *et al*., 1997), YdeO might upregulate other acid resistance genes. The EvgA-induced acid resistance is reduced by deletion of *ydeP* or *ydeO*, and is completely abolished by deletion of a putative *ydeP–b1500–ydeO* operon (Masuda and Church, 2002). The reduction in EvgA-induced acid resistance by *ydeO* deletion independently of *ydeP* deletion suggests that YdeO induces gene(s) involved in acid resistance except for *ydeP*. The reduction in EvgA-induced acid resistance in the *yhiE* deletion strain suggests that *yhiE* is a candidate of YdeO-induced acid resistance gene. To test this hypothesis, we introduced pQEydeO into the *yhiE* and *ydeP* deletion strains by electroporation and tested acid resistance. Overexpression of YdeO conferred acid resistance to the *ydeP* deletion strain but not the *yhiE*

deletion strain (Fig. 1, bars 5–8). These results suggest that *yhiE* is essential for acid resistance induced by YdeO overexpression and that EvgA overexpression induces *yhiE* via the induction of YdeO. In addition, EvgA overexpression did not confer acid resistance to a Δ*yhiE*Δ*ydeP* strain (data not shown). These results are similar to our previous observation that EvgA overexpression does not confer acid resistance to the *ydeP-b1500-ydeO* deletion strain, and are consistent with the hypothesis that YdeO induces *yhiE* expression.

YdeO and YhiE overexpression had no effect on growth rate, whereas EvgA and YdeP overexpression reduced growth rate (data not shown). Overexpression of another AraC/XylS family transcriptional regulator, GadX (described below), also reduced the growth rate, but did not confer acid resistance (data not shown). The relationship between acid resistance and reduction in growth rate caused by YdeP overexpression remains unclear.

EvgA regulon includes YdeO regulon

We have identified 37 EvgA-activated genes by comparison of mRNA levels in EvgA-overexpressing strains with those in EvgA-lacking strains using oligonucleotide microarrays (Masuda and Church, 2002). We hypothesized that increased levels of YdeO might induce some EvgA-activated genes. To identify such YdeO-dependent genes, we analysed the transcript profiles of Δ*ydeO* and Δ*evgAS*Δ*ydeO* strains bearing pQEevgA, an EvgA expression plasmid, grown in LB broth with or without 1 mM IPTG. Fifty-four open reading frames (ORFs) showed a significant increase in transcription, and 20 ORFs showed a significant decrease in transcription. Of the 54 upregulated ORFs, 13 ORFs showed a more than fourfold increase ($\log_2$ ratio >2) in both experiments (Table 1). The 13 highly upregulated ORFs included *evgA*, the gene overexpressed by the plasmid itself. We added *b1500* and *emrK* to Table 1 because they were highly and significantly induced in one of the two experiments and located in putative operons with other highly and significantly induced genes. Twelve of the 15 ORFs listed in Table 1 were also highly induced by EvgA overexpression in the presence of *ydeO* (Masuda and Church, 2002). In a previous study, we eliminated *emrY* from the list of highly upregulated genes by EvgA overexpression because of marginally significant *P*-values in three out of four experiments, even though the expression of *emrY* was induced 4.6-fold on average. However, these marginally significant *P*-values are likely to be the result of experimental error because *emrK* and *emrY* apparently form the *emrKY* operon, which is regulated by EvgAS (Kato *et al*., 2000). Therefore, we left *emrY* on the list for further consideration in this study, and eliminated the other two ORFs that showed increased transcription only in the absence of *ydeO*. Only one of the 20 downregulated ORFs showed a more than fourfold decrease ($\log_2$ ratio of <−2) in both experiments. Because we were primarily interested in genes induced by EvgA overexpression, we did not give further consideration to the gene repressed by EvgA overexpression.

To identify YdeO-regulated genes, the comprehensive transcript profiles of the Δ*ydeO* strain bearing plasmid

Table 1. Genes highly increased by EvgA overexpression in the absence of *ydeO*.

			$\log_2$ ratio[a]	
b no.	Gene[b]	Gene description[c]	Expt 1	Expt 2
b1500	*b1500*[d]	ORF, hypothetical protein	3.4	8.9
b1501	*ydeP*	Putative oxidoreductase, major subunit	4.7	9
b2082	*ogrK*[e]	Prophage P2 ogr protein	3.2	2.5
b2083	*yegZ*	ORF, hypothetical protein	6.4	6.9
b2084	*b2084*	ORF, hypothetical protein	2.5	4.5
b2085	*yegR*	ORF, hypothetical protein	5.2	6.4
b2367	*emrY*[e]	Multidrug resistance protein Y	4	4.5
b2368	*emrK*[d]	Multidrug resistance protein K	0.9	5.6
b2369	*evgA*	Putative positive transcription regulator	4.9	6.8
b2371	*yfdE*	Putative enzyme	6.3	6.5
b2372	*yfdV*	Putative receptor protein	6.3	6.9
b2373	*yfdU*	Putative enzyme	7.7	7.1
b2374	*yfdW*	Putative enzyme	6.2	10.4
b2375	*yfdX*	ORF, hypothetical protein	8.8	6
b3686	*ibpB*[e]	Heat shock protein	6.3	3.3

a. Indicates the $\log_2$ ratio of transcript levels in the presence of IPTG versus those in the absence of IPTG in Δ*ydeO*/pQEevgA (Expt 1) and Δ*evgAS*Δ*ydeO*/pQEevgA (Expt 2) respectively.
b. Gene names are taken from http://bmb.med.miami.edu/EcoGene/EcoWeb/.
c. Gene descriptions are taken from the Affymetrix Expression Analysis Sequence Information Database.
d. Gene listed although one of two change calls was 'no change' (see text for details).
e. Gene not highly and significantly increased by EvgA overexpression in the previous study (Masuda and Church, 2002).

Table 2. Genes highly increased by YdeO overexpression.

b no.	Gene[b]	Gene description[c]	Log_2 ratio[a] Expt 1	Expt 2
b0791	*ybhQ*[d]	ORF, hypothetical protein	2.5	2.8
b0978	*appC*	Probable third cytochrome oxidase, subunit I	5.6	3.7
b0979	*appB*	Probable third cytochrome oxidase, subunit II	4.4	6
b1330	*ynaI*	ORF, hypothetical protein	3.5	3
b1492	*gadC*	Acid sensitivity protein, putative transporter	5.3	5.5
b1493	*gadB*	Glutamate decarboxylase isozyme	5.6	8.1
b1499	*ydeO*	Putative AraC-type regulatory protein	6	6.1
b2418	*pdxK*[d]	Pyridoxal/pyridoxine/pyridoxamine kinase	3.3	3.7
b3506	*slp*	Outer membrane protein induced after carbon starvation	6.5	7.5
b3507	*yhiF*	ORF, hypothetical protein	3.7	3.1
b3508	*yhiD*[e]	Putative transport ATPase	1.7	3.5
b3509	*hdeB*	ORF, hypothetical protein	7.2	8.2
b3510	*hdeA*	ORF, hypothetical protein	7.4	5.7
b3511	*hdeD*	ORF, hypothetical protein	3.1	5.4
b3512	*yhiE*	ORF, hypothetical protein	6.8	6.5
b3513	*yhiU*	Putative membrane protein	2.1	2.6
b3514	*yhiV*	Putative transport system permease protein	3	2.5
b3517	*gadA*	Glutamate decarboxylase isozyme	6.5	8.5
b3922	*yiiS*	ORF, hypothetical protein	2.2	3.1
b4113	*basR*	Transcriptional regulatory protein	3.1	2.5
b4114	*yjdB*[d]	ORF, hypothetical protein	3.7	4.7
b4115	*yjdE*	Putative amino acid/amine transport protein	5.5	5.4
b4117	*adiA*[d]	Biodegradative arginine decarboxylase	4.2	3.9

a. Indicates the log_2 ratio of transcript level in the presence of IPTG versus that in the absence of IPTG in Δ*ydeO*/pQEydeO. Expt 1 and Expt 2 are two independent experiments.
b. Gene names are taken from http://bmb.med.miami.edu/EcoGene/EcoWeb/.
c. Gene descriptions are taken from the Affymetrix Expression Analysis Sequence Information Database.
d. Gene not highly and significantly increased by EvgA overexpression in the previous study (Masuda and Church, 2002).
e. Gene listed although one of two log_2 ratio was not >2 (see text for details).

pQEydeO grown in the presence of 1 mM IPTG were also compared with those grown in the absence of IPTG. These experiments were performed in duplicate and identified 51 ORFs showing a significant increase in transcription and 53 ORFs showing a significant decrease in transcription. Of the 51 upregulated ORFs, 22 showed a more than fourfold increase in both experiments (Table 2), whereas only two of the 53 downregulated ORFs showed a more than fourfold decrease in both. The 22 highly upregulated ORFs included *ydeO*, overexpressed by the plasmid itself, and *yhiE*. We added *yhiD* to Table 2 because it was significantly induced in both experiments, highly induced in one of the two experiments and located in a putative operon with other highly and significantly induced genes. Nineteen of the 23 ORFs listed in Table 2 were also highly induced by EvgA overexpression (Masuda and Church, 2002). We eliminated the other four genes from further consideration, as these genes might be false positives or induced only by the higher level of YdeO relative to the YdeO levels induced by EvgA overexpression.

Altogether, microarray analysis allowed the identification of 12 genes induced by EvgA overexpression in the *ydeO* deletion strain, *ydeP-b1500*, *yegR-b2084-yegZ*, *emrKY* and *yfdXWUVE* (Table 1). Microarray analysis also identified 18 YdeO-activated genes, *appCB*, *ynaI*, *gadBC*, *slp-yhiF*, *hdeAB-yhiD*, *hdeD-yhiEUV*, *gadA*, *yiiS*, *basR* and *yjdE* (Table 2). This latter set of genes was induced by EvgA overexpression in the presence of *ydeO* (Masuda and Church, 2002), but not by EvgA overexpression in the absence of *ydeO* (http://arep.med.harvard.edu/cgi-bin/ExpressDBecoli/EXDStart). These results suggest that EvgA directly regulates the former genes, and indirectly regulates the latter genes via YdeO.

EvgA-binding motif

To identify the EvgA-binding motif, we analysed the upstream regions of the genes induced by EvgA overexpression in the *ydeO* deletion strain. We used the upstream regions of the first gene in each putative operon, *ydeP*, *yegR*, *emrK*, *yfdW* and *yfdX*, for analysis. We aligned 500 bp fragments (from 400 bp upstream to 100 bp downstream of the start codon) with the motif-finding program ALIGNACE (Roth *et al.*, 1998). The algorithm identified a common 14 bp sequence motif in all the input sequences. We extended this to an 18 bp sequence motif by searching the sequences adjacent to the 14 bp sequences manually. A SCANACE search of the *E. coli* genome (Robison *et al.*, 1998) identified an additional

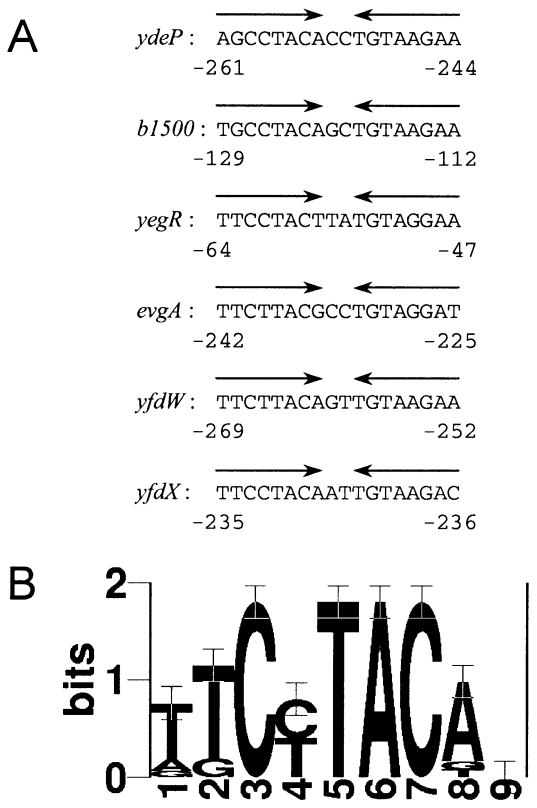

Fig. 2. Consensus sequence weight matrix for EvgA binding.
A. Sequences found upstream of the genes induced by EvgA overexpression in the *ydeO* deletion strain. Arrows indicate the inverted repeats. The numbering is relative to the start codon of the genes listed.
B. Sequence logo (Schneider and Stephens, 1990) for half the inverted repeat motifs (combining data from both halves). The height of each letter is related to its frequency at that position. The overall height of the stack at each position represents the informational content of that position in bits of information ranging from 0 to 2 bits.

18 bp consensus sequence in the upstream region of *b1500*, suggesting that *b1500* and *ydeO* form an operon and that they are transcribed independently of *ydeP*. Altogether, analysis with the motif-finding programs ALIGNACE and SCANACE identified six 18 bp consensus sequences in upstream regions of *ydeP*, *b1500*, *yegR*, *emrK* (*evgA*), *yfdX* and *yfdW* (Fig. 2). The 18 bp consensus sequence consists of 5′-TTCPyTACA-3′ and its inverted repeat 5′-TGTAPuGAA-3′ separated by two random bases. In addition, the SCANACE search identified a group of 18 bp sequences that are similar to the motif but have one or two substituted bases at highly conserved bases in the motif. However, most of these sequences are located in coding regions, with only two located in intergenic regions, upstream of *yqjI* and *ugpB*. EvgA overexpression had no effect on the expression of *yqjI* and *ugpB* (http://arep.med.harvard.edu/cgi-bin/ExpressDBecoli/EXDStart). These results suggest that EvgA binds only the six 18 bp consensus sequences.

To confirm the function of the 18 bp motif in EvgA activity, we introduced transversion mutations into the predicted EvgA-binding sequences upstream of *ydeP* and *b1500* and assessed the expression of *ydeP* and *ydeO* by performing a reporter assay. First, the predicted EvgA-binding sequence, 5′-AGCCTACACCTGTAAGAA-3′, upstream of *ydeP* was replaced with 5′-AGaCgcaACCT tgcAtAA-3′ (lower case letters indicate mutated bases), and 5′-TGCCTACAGCTGTAAGAA-3′ upstream of *b1500* was replaced with 5′-TGaCgcaAGCTtgcAtAA-3′ by allelic exchange. These mutants were designated BMydeP and BM1500 respectively. We also constructed the double mutant and designated it BMydeP1500. Secondly, the ORF of *ydeP* or *ydeO* was completely replaced by that of *bla* by allelic exchange. Finally, the constructed strains were transformed with pHSGevgA, a constitutive EvgA expression plasmid harbouring a chloramphenicol-resistant gene. The mutation in the predicted binding sequence upstream of *ydeP* completely abolished the expression of *bla* introduced at *ydeP*, and slightly reduced the expression of *bla* introduced at *ydeO* (Fig. 3). The mutation in the predicted binding sequence upstream of *b1500* drastically reduced the expression of *bla* introduced at *ydeO*, although slight expression was still detected. Mutating both predicted binding sequences completely abolished the expression of *bla* introduced at *ydeO*. These results indicate that both 18 bp motifs are necessary for *ydeP* and *ydeO* induction by EvgA overexpression.

We also transformed BMydeP, BM1500 and BMydeP1500 with pQEevgA and tested acid resistance

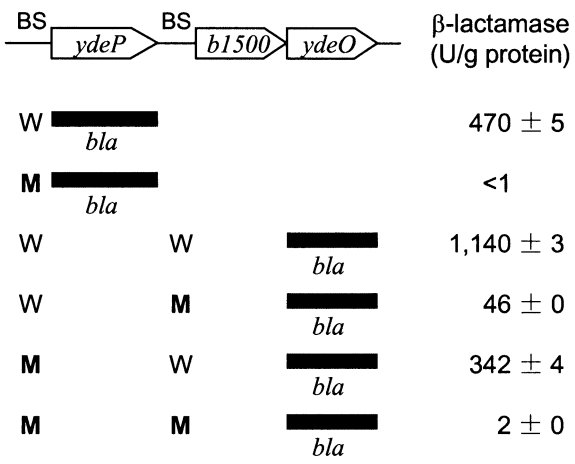

Fig. 3. Effect of mutations at predicted EvgA-binding sequences on *ydeP* and *ydeO* expression. The pHSGevgA-harbouring strains that have wild-type (W) or mutated (M) binding sequences (BS) upstream of *ydeP* and *b1500* were grown to mid-log phase in LB broth (pH 7.0) and examined for β-lactamase activity expressed from *bla* introduced at *ydeP* or *ydeO* (replaced region of each strain is expressed as a bar). Experiments were performed in triplicate, and β-lactamase activity is represented as mean value ± standard error of the mean.

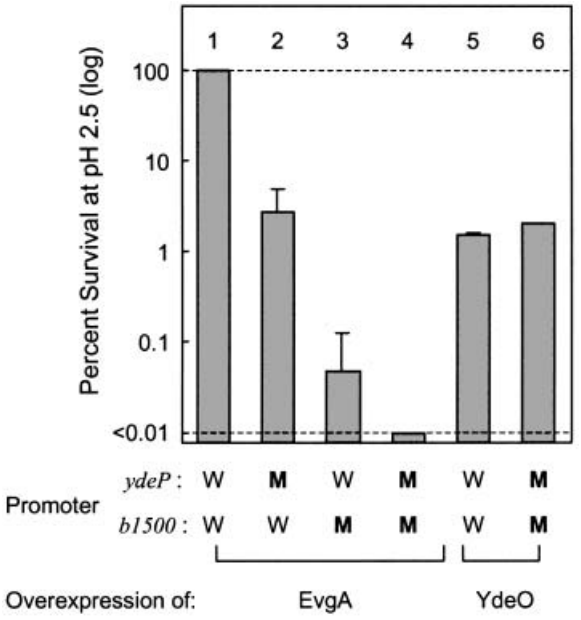

Fig. 4. Acid survival of strains with mutations at predicted EvgA-binding sequences. MG1655 (lanes 1 and 5), BMydeP (lane 2), BM1500 (lane 3) and BMydeP1500 (lanes 4 and 6) harbouring pQEevgA (lanes 1–4) and pQEydeO (lanes 5 and 6) were grown to mid-log phase in LB broth (pH 7.0) containing 1 mM IPTG. Cells were diluted 40-fold into LB broth (pH 2.5) and incubated for 1 h at 37°C. Initial cell densities ranged from 2.0×10^6 to 8.9×10^6 cfu ml^{-1}. Error bars represent standard errors of the mean.

induced by EvgA overexpression. Both mutations resulted in a partial reduction in acid resistance, although the effect of the mutation upstream of *b1500* was more severe (Fig. 4, bars 1–3). In addition, mutating both motifs completely abolished acid resistance (Fig. 4, bar 4). These results are consistent with the effects of the *ydeP* and *b1500* deletions reported previously (Masuda and Church, 2002). In contrast, the introduction of both mutations had no effect on acid resistance induced by YdeO overexpression (Fig. 4, bars 5–6). Taken together, these results demonstrate that both 18 bp motifs are necessary for EvgA-dependent acid resistance and suggest that EvgA binds to the 18 bp motif.

To test whether EvgA could interact with the 18 bp sequences directly, we performed gel mobility shift assays. We used nine 36 bp DNA probes, the six consensus sequences, the two similar sequences upstream of *yqjI* and *ugpB* and a similar sequence located in the *yhiU* ORF. Each probe consisted of the 18 bp binding site flanked on each side by 9 bp. Cy3-labelled probes corresponding to the six consensus sequences produced bandshifts when incubated with His-tagged EvgA (Fig. 5). Excess amount (×50) of unlabelled probe corresponding to each consensus sequence competed the binding, whereas the unlabelled yqjI, ugpB and yhiU probes did not. Therefore, interactions between His-tagged EvgA and the six consensus sequences are specific. These results are consistent with the microarray data and suggest that EvgA binds to the six consensus sequences in the genome to induce the expression of *ydeP*, *b1500-ydeO*, *yegR-b2084-yegZ*, *emrKY*, *evgAS*, *yfdX* and *yfdWUVE*.

Overexpression of EvgA induces the efflux pump genes *yhiUV* and confers multidrug resistance to a strain that lacks the major efflux pump genes *acrAB* (Masuda and Church, 2002; Nishino and Yamaguchi, 2002). Nishino and Yamaguchi (2002) have reported that EvgA binds directly to the *yhiU* promoter regions. However, our results suggest that there is no EvgA-binding motif in the upstream region of *yhiU*, and that EvgA induces *yhiUV* indirectly via YdeO. To confirm the involvement of YdeO in EvgA-induced multidrug resistance, a Δ*acrB*Δ*ydeO* strain was constructed from an *acrB* deletion strain and

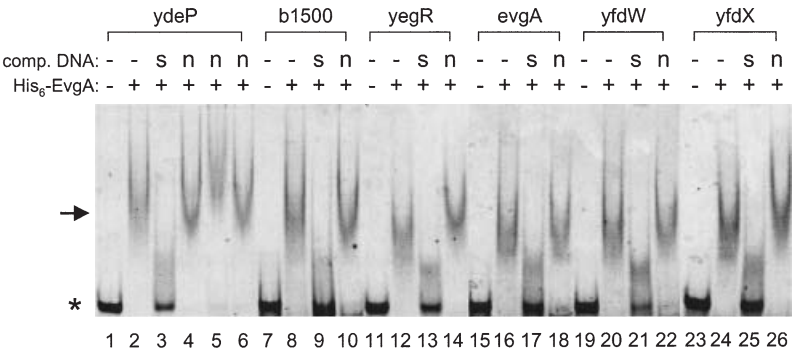

Fig. 5. Gel mobility shift assay showing competition for binding of His-tagged EvgA to Cy3-labelled probe DNAs by specific (s) and non-specific (n) competitor DNA. Cy3-labelled probes, described in Table 5, are given at the top. The probes were incubated with (+) or without (–) His-tagged EvgA and loaded on 6% polyacrylamide–0.5× Tris borate–EDTA native gel. The DNA-containing band positions were visualized using a Bio-Rad Molecular Imager FX system. Excess unlabelled DNA corresponding to each labelled probe (lanes 3, 9, 13, 17, 21 and 25), unlabelled yqjI (lanes 4, 10, 14, 18, 22 and 26), ugpB (lane 5) or yhiU (lane 6) described in Table 5 was added to some reaction mixtures. Arrows indicate the position of migration of complex formed between His-tagged EvgA and the DNA probes; asterisks indicate the position of migration of the unbound DNA probes.

Table 3. Drug susceptibilities of constructed strains.

Strain	IPTG[b]	MIC (μg ml^{-1}) of[a]: ERY	SDS	R6G
Δ*acrB*/pQEevgA	–	4	125	8
Δ*acrB*/pQEevgA	+	64	>16 000	128
Δ*acrB*Δ*ydeO*/pQEevgA	–	4	125	8
Δ*acrB*Δ*ydeO*/pQEevgA	+	4	125	8
Δ*acrB*/pQEydeO	–	4	125	8
Δ*acrB*/pQEydeO	+	128	>16 000	256
Δ*acrB*Δ*evgAS*/pQEydeO	+	128	>16 000	256
Δ*acrB*Δ*hdeA-D*/pQEydeO	+	128	>16 000	256
Δ*acrB*Δ*hdeD-yhiE*/pQEydeO	+	4	62.5	8

a. ERY, erythromycin; SDS, sodium dodecyl sulphate; R6G, rhodamine 6G.
b. MICs were determined in the absence (–) or presence of 1 mM IPTG.

transformed with pQEevgA. We also introduced pQEydeO into the Δ*acrB* and Δ*acrB*Δ*evgAS* strains by transformation and tested their drug susceptibilities in the presence of 1 mM IPTG. The multidrug resistance in the Δ*acrB* strain caused by EvgA overexpression was completely abolished by deletion of *ydeO* (Table 3). Overexpression of YdeO conferred multidrug resistance to the Δ*acrB* and Δ*acrB*Δ*evgAS* strains (Table 3). These results suggest that YdeO is essential for YhiUV induction by EvgA, consistent with our EvgA-binding motif analysis.

Comparison of YdeO and GadX regulons

It has been reported that overexpression of GadX, another member of the AraC/XylS transcriptional regulators, induces *gadBC*, *hdeD* and *gadA* (Hommais *et al*., 2001; Shin *et al*., 2001; Tramonti *et al*., 2002), which were also induced by YdeO overexpression (Table 2). Hommais *et al*. (2001) compared the proteome and transcriptome of an H-NS-deficient strain and its parent strain, and reported that the expression of *evgA* and *gadX* is induced by deletion of a nucleoid protein H-NS. These results suggest the presence of a complex regulatory network involving H-NS, EvgA, GadX and YdeO. To identify GadX-regulated genes, we constructed a GadX expression plasmid and a *gadX* deletion strain, and compared the comprehensive transcript profiles of the Δ*gadX* strain bearing plasmid pQEgadX grown in the presence of 0.1 mM IPTG with those grown in the absence of IPTG. It has been reported that high levels of GadX overexpression are detrimental to cell viability (Tramonti *et al*., 2002). Because MG1655 cells harbouring pQEgadX showed no growth in the presence of 1 mM IPTG (data not shown), we used 0.1 mM IPTG for GadX induction. In duplicate experiments, 22 ORFs showed a significant increase in transcription, and 35 ORFs showed a significant decrease in transcription. Of the 22 upregulated ORFs, eight ORFs showed a more than fourfold increase in at least one of the two experiments (Table 4). In this case, we used different criteria to select highly induced genes because the concentration of the inducer was lower than in the other experiments. The eight highly upregulated ORFs are *yafN*, *gadB*, *ydgTK*, *nanA*, *yhiO*, *slp* and *gadX*, which is overexpressed by the plasmid itself. Two other genes, *hdeD* and *gadA*, were added to Table 4 because they were highly induced in one of the two experiments and reported to be induced by GadX overexpression. Only two of the 35 downregulated ORFs showed a more than fourfold decrease in at least one of the two experiments.

GadX overexpression conferred no acid resistance to exponentially growing cells in LB broth (data not shown). Several of the genes induced by the overexpression of YdeO and GadX were the same. However, YdeO overex-

Table 4. Genes highly increased by GadX overexpression.

b no.	Gene[b]	Gene description[c]	Log_2 ratio[a]: Expt 1	Expt 2
b0232	*yafN*	ORF, hypothetical protein	1.5	2.6
b1493	*gadB*	Glutamate decarboxylase isozyme	4.6	3.4
b1625	*ydgT*	ORF, hypothetical protein	1.9	2.3
b1626	*ydgK*	ORF, hypothetical protein	1.2	3.3
b3225	*nanA*	*N*-acetylneuraminate lyase (aldolase)	2.3	1.2
b3494	*yhiO*	ORF, hypothetical protein	3.8	3
b3506	*slp*	Outer membrane protein induced after carbon starvation	1.9	2.5
b3511	*hdeD*[d]	ORF, hypothetical protein	2.2	1.7
b3516	*gadX*	Putative AraC-type regulatory protein	5.1	4.3
b3517	*gadA*[d]	Glutamate decarboxylase isozyme	1.6	3.2

a. Indicates the log_2 ratio of transcript level in the presence of IPTG versus that in the absence of IPTG in Δ*gadX*/pQEgadX. Expt 1 and Expt 2 are two independent experiments.
b. Gene names are taken from http://bmb.med.miami.edu/EcoGene/EcoWeb/.
c. Gene descriptions are taken from the Affymetrix Expression Analysis Sequence Information Database.
d. Gene listed although one of two change calls was 'no change' (see text for details).

pression induced *yhiE*, whereas GadX overexpression did not. The inability of GadX to induce *yhiE* might explain its lack of acid resistance induction in exponentially growing cells. The expression of *yhiE* is induced 52-fold in stationary phase (Selinger *et al*., 2000). Hommais *et al*. (2001) have reported that GadX overexpression confers acid resistance to stationary phase cells in M9 medium supplemented with 0.012% glutamate. The varying levels of YhiE might explain the discrepancy between the effects of GadX overexpression on acid resistance in stationary and exponential phases.

The *hdeD* and *yhiEUV* genes are located in tandem, and they might be co-transcribed. However, the fact that YdeO induced *yhiE* but GadX did not suggests that YdeO binds to the downstream region of *hdeD*. To check this possibility, we constructed an Δ*acrB*Δ*hdeA-hdeD* strain and an Δ*acrB*Δ*hdeD-yhiE* strain, and transformed them with pQEydeO. Overexpression of YdeO conferred multidrug resistance to the Δ*acrB*Δ*hdeA-hdeD* strain but not to the Δ*acrB*Δ*hdeD-yhiE* strain (Table 3). These results suggest that YdeO binds to the downstream region of *hdeD*, upregulates *yhiUV* and, consequently, induces multidrug resistance in a Δ*acrB* strain.

Genes involved in YdeO-induced acid resistance

Our previous analysis demonstrated that a *ydeO* deletion reduced EvgA-induced acid resistance significantly more than a *yhiE* deletion (Masuda and Church, 2002). In this study, we show that YdeO overexpression induces a higher level of acid resistance than YhiE overexpression (Fig. 1, bars 3 and 4). These results suggest that YdeO induces acid resistance gene(s) in addition to *yhiE*. To identify additional gene(s) responsible for the acid resistance caused by YdeO overexpression, we introduced pQEydeO by electroporation into the deletion mutants of *appCBA*, *gadC*, *slp-yhiF*, *yhiD*, *hdeB*, *hdeA*, *hdeD*, *yhiUV*, *yiiS* and *yjdE*, which were induced by YdeO overexpression, and tested for acid resistance. Exponential phase cultures of each strain harbouring pQEydeO grown in LB broth (pH 7.0) containing 1 mM IPTG were incubated in acidic LB broth (pH 2.5) for 1 h, and percentage survival values were determined as described in *Experimental procedures*. In MG1655 cells harbouring pQEydeO, 1.5% of the cells survived after 1 h exposure to acidic conditions. The deletions of *gadC*, *hdeA* and *hdeD* decreased this survival rate to <0.01%, and the deletion of *slp-yhiF* decreased survival to 0.015% (Fig. 6, bars 3, 4, 7 and 8). These results suggest that, in addition to *yhiE*, *gadC*, *slp-yhiF*, *hdeA* and *hdeD* are essential for YdeO-induced acid resistance.

Slp is an outer membrane lipoprotein induced during carbon starvation and stationary phase (Alexander and St John, 1994). YhiF is another LuxR superfamily member that has 24% sequence identity with YhiE. YhiF is thought to act as a regulator of *dctA*, a gene involved in dicarboxylate transport (Boogerd *et al*., 1998). The precise functions of YhiE, Slp, YhiF and HdeD are unknown. The deletions of *yhiD* and *hdeB* decreased survival from 1.5% to 0.23% and 0.093%, respectively (Fig. 6, bars 5 and 6), whereas the deletions of the other genes had little effect on the 1.5% survival rate. The contributions of *yhiD* and *hdeB* to YdeO-induced acid resistance are unclear. Although *appCBA*, *yhiUV*, *yiiS* and *yjdE* do not seem to be involved in YdeO-induced acid resistance, we cannot exclude the possibility that these genes are involved in acid resistance but their contributions are redundant and, thus, we were not able to detect change in acid resistance by single deletions. Multiple deletion experiments will be necessary to elucidate the involvement of such genes.

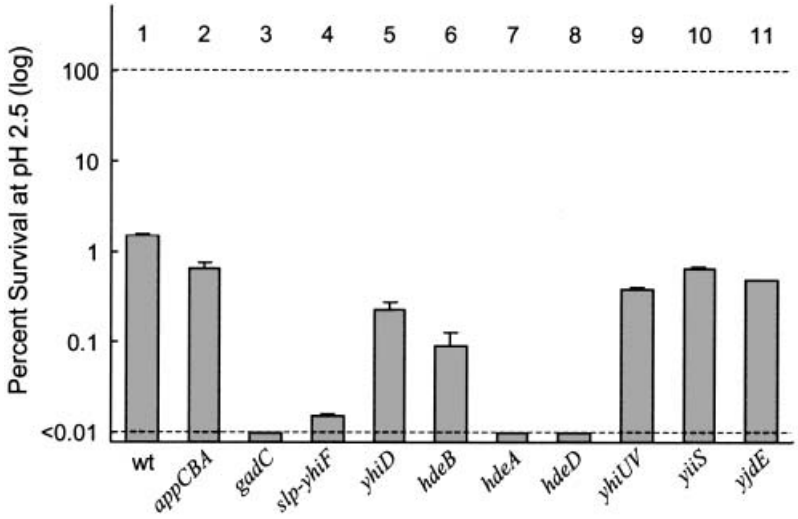

Fig. 6. Acid survival of YdeO overexpression strains. MG1655 (wt) and various deletion mutants harbouring pQEydeO were grown to mid-log phase in LB broth (pH 7.0) containing 1 mM IPTG. Cells were diluted 40-fold into LB broth (pH 2.5) and incubated for 1 h at 37°C. Initial cell densities ranged from 5.0×10^6 to 9.0×10^6 cfu ml^{-1}. Error bars represent standard errors of the mean.

A model for regulatory network of acid resistance genes

In this study, we have identified genes directly upregulated by EvgA and genes upregulated by EvgA-induced YdeO. We have also identified six EvgA-binding sequences using a combination of computational and genetic techniques. Many of the YdeO-activated genes were also activated by GadX, whereas *yhiEUV* were induced only by YdeO. Our results suggest that several acid resistance genes orchestrate YdeO-induced acid resistance. The expression of the *gad* system and *hdeAB* is repressed by a nucleoid protein H-NS during the exponential growth phase (Yoshida *et al*., 1993a,b,c; De Biase *et al*., 1999). H-NS also represses the expression of *evgA* and *gadX* (Hommais *et al*., 2001). Therefore, we propose the following model for the regulatory network of the acid resistance genes (Fig. 7). H-NS negatively regulates *evgA* and *gadX*. Based on our findings, EvgA binds six consensus sequences and induces

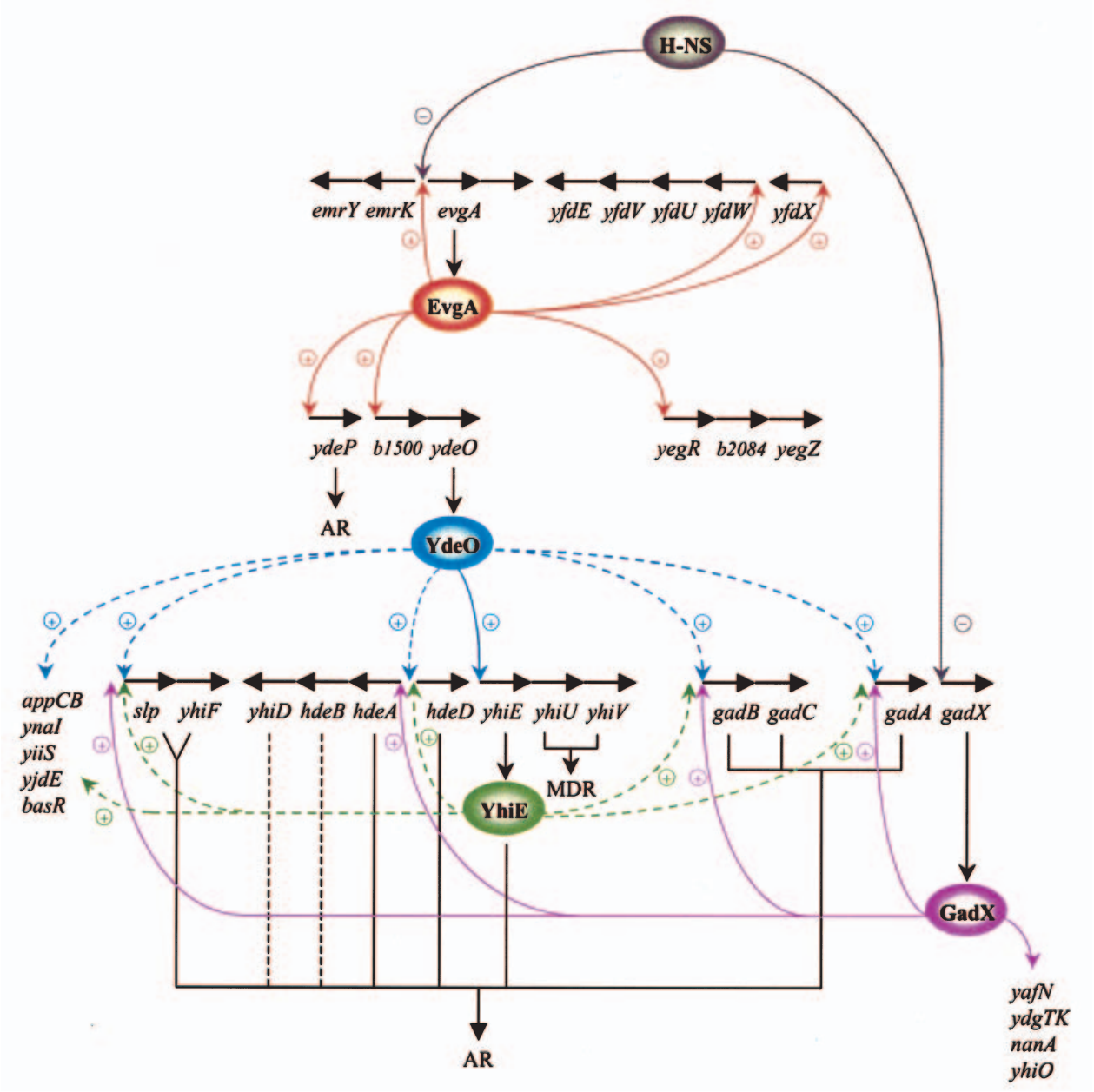

Fig. 7. Regulatory network of acid resistance genes. YdeO-regulated Slp-YhiF, HdeA, HdeD, YhiE and GadABC induce acid resistance (AR) by working co-operatively. The contributions of YhiD and HdeB to the AR is unclear. It is also unclear whether YdeO induces *slp-yhiF*, *hdeAB-yhiD*, *hdeD*, *gadA* and *gadBC* directly or via YhiE. EvgA-regulated YdeP induces AR. YdeO-regulated YhiUV induce multidrug resistance (MDR). GadX activates some of the YdeO-regulated genes but does not activate *yhiEUV*. +, activation; –, inhibition.

seven operons (genes) including *ydeO* and *ydeP*. YdeP induces acid resistance by an unknown mechanism. YdeO induces Slp-YhiF, HdeA, HdeD, YhiE and GadABC, which induce acid resistance by working co-operatively. YhiE, a member of the LuxR superfamily, instead of YdeO itself, might directly induce some YdeO-activated genes. The contributions of YhiD and HdeB to acid resistance are unclear. YdeO also induces YhiUV, which induce multidrug resistance. GadX activates some of the YdeO-regulated genes but does not activate *yhiEUV*. There is a hierarchical regulation of the genes that are necessary to accomplish acid resistance in *E. coli*.

Although we reported low-level induction of *gadX* by EvgA overexpression (Masuda and Church, 2002), we could not detect *gadX* induction by either EvgA overexpression in the absence of *ydeO* or YdeO overexpression (http://arep.med.harvard.edu/cgi-bin/ExpressDBecoli/EXDStart). Although *gadX* is predicted to be co-transcribed with *gadA* (Tramonti *et al*., 2002), EvgA overexpression in the absence of *ydeO* or YdeO overexpression upregulated *gadA* but not *gadX*. The reason for these discrepancies is unclear. GadX has been thought to play a central role in the H-NS control of *gadA* and *gadBC* (Hommais *et al*., 2001; Tramonti *et al*., 2002). In contrast, Tramonti *et al*. (2002) also suggested that *gadX* and additional positive regulators are involved in the transcriptional tuning of the *gad* system. Our results suggest that the *gad* system is positively regulated by at least two AraC/XylS-type regulators, YdeO and GadX, and also suggest that EvgA and YdeO regulate acid resistance genes more globally than GadX. De Biase *et al*. (1999) concluded that the *gad* system is necessary for the acid survival of *E. coli*, but insufficient alone to confer the acid resistance phenotype. The acid resistance phenotype must be attained by the co-operation of various proteins, such as GadABC, HdeA and YhiE.

Spory *et al*. (2002) reported that *hdeAB*, *gadA*, *slp* and *yhiU* are induced by the transcriptional regulator SlyA. Iyer *et al*. (2002) reported that either *eriC* (*yadQ*) or *mriT* (*ynfJ*), which encode ClC chloride channels, is necessary for both glutamate and arginine decarboxylase systems. Ma *et al*. (2002) reported that GadW (YhiW) is another regulator of *gadA* and *gadBC*. However, EvgA, YdeO and GadX overexpression had no effect on *slyA*, *eriC*, *mriT* and *gadW* expression (http://arep.med.harvard.edu/cgi-bin/ExpressDBecoli/EXDStart). These results suggest that there is a more complex system that regulates the genes related to acid resistance.

In this study, we have proposed a model for the regulatory network of several *E. coli* genes involved in acid resistance. However, the input signal needed to activate this network remains unclear. EvgA is one part of a two-component system. In *Bacillus subtilis*, the overproduction of the unphosphorylated response regulators resulted in altered expression of the target genes in the absence of the environmental signals responsible for their phosphorylation (Ogura *et al*., 2001). It is not known whether EvgA phosphorylation leads to activation of the network and whether change in the expression level of EvgA plays a physiological role in *E. coli*. Several groups have characterized the *gad* system, whereas the functions of YdeP, YhiE, Slp, YhiF and HdeD remain unclear. In addition, various media compositions and pH levels have been used for acid resistance assay, and these conditions are likely to affect the results. Further study is necessary to evaluate our model and to elucidate the complete mechanism of acid resistance. The series of deletion mutants and expression plasmids constructed in this study will be useful for such analysis.

Experimental procedures

Bacterial strains, plasmids and media

Strains and plasmids used in this study were described in a previous report (Masuda and Church, 2002) or in this study. Bacterial cells were grown in Luria–Bertani (LB) broth (pH 7.0) or on LB agar medium (BIO 101). Antibiotics were added, when required, at the following final concentrations: 100 μg ml^{-1} carbenicillin and 20 μg ml^{-1} chloramphenicol for AcrB-producing strains and 5 μg ml^{-1} chloramphenicol for AcrB-deficient strains. LB agar was supplemented with 10% (w/v) sucrose, as required.

Molecular biological techniques

Chromosomal DNA was isolated with a DNeasy tissue kit (Qiagen). Plasmids were isolated with a HiSpeed plasmid purification kit or a QIAprep Miniprep kit (Qiagen). Polymerase chain reaction (PCR) for plasmid construction was performed using the high-fidelity platinum *Pfx* DNA polymerase (Invitrogen). Diagnostic PCRs were performed with *Z-Taq* polymerase (Panvera). PCR products were purified with a QIAquick PCR purification kit (Qiagen). Restriction endonucleases and alkaline phosphatase were obtained from New England Biolabs. The DNA ligation kit was obtained from Panvera. Restriction fragments were purified with a QIAquick PCR purification kit or a MinElute reaction cleanup kit (Qiagen). All molecular biology techniques were carried out according to the manufacturer's instructions or as described by Sambrook *et al*. (1989).

Construction of plasmids for gene expression

YdeO, YdeP, YhiE and GadX expression plasmids, pQEydeO, pQEydeP, pQEyhiE and pQEgadX, respectively, were constructed as follows. Full-length ORFs except for start codons were amplified by PCR from MG1655 with the primer pairs listed in Table 5. These primers also introduced restriction sites at the 5′ and 3′ ends of the PCR-generated gene, enabling the amplified gene to be inserted into the restriction sites of the expression vector pQE80L (Qiagen) in the correct

Table 5. Oligonucleotides used for plasmid construction, verification of gene replacement and gel mobility shift assay.

Oligonucleotide	Oligonucleotide sequence (5′ to 3′)[a]	Enzyme[b]
pQEydeO-N	cgcggatccTCGCTCGTTTGTTCTGTTATATTTATTCATCATG	*Bam*HI
pQEydeO-C	ttccttctgcagTCAAATAGCTAAAGCATTCATCGTGTTGC	*Pst*I
pQEydeP-N	cgcggatccAAGAAAAAAATTGAATCCTACCAGGGTG	*Bam*HI
pQEydeP-C	cgcacgcatgtcgacTTAATTTGATGGTTCTAATTCAACCGGAATAC	*Sal*I
pQEyhiE-N	cgcggatccATTTTTCTCATGACGAAAGATTCTTTTCTTTTACAG	*Bam*HI
pQEyhiE-C	ttccttctgcagCTAAAAATAAGATGTGATACCCAGGGTGAC	*Pst*I
pQEgadX-N	cgcggatccCAATCACTACATGGGAATTGTCTAATTGC	*Bam*HI
pQEgadX-C	ttccttctgcagCTATAATCTTATTCCTTCCGCAGAACGG	*Pst*I
pKOhdeA-D-No	aaggaaaaaagcggccgcTGCTGCTTAAACCAGTCGAGC	*Not*I
pKOhdeA-D-Ni	caaccggggtaattaTAATTCCGGGAATGCGTTACATCG	
pKOhdeA-D-Ci	gcattcccggaattaTAATTACCCCGGTTGTCACCC	
pKOhdeA-D-Co	cgcacgcatgtcgacTTCTCTCGGCATCTAATTTCTCCAG	*Sal*I
pKOgadX-No	aaggaaaaaagcggccgcGATATCGTCTGGGACTTCCGC	*Not*I
pKOgadX-Ni	ttatgtctgagtaaaactctaACAATTCCCATGTAGTGATTGCATAGTTG	
pKOgadX-Ci	cactacatgggaattgtTAGAGTTTTACTCAGACATAAAAAAAACCCG	
pKOgadX-Co	cgcacgcatgtcgacGCCACATTTTTCCGCAATAGTATGC	*Sal*I
pBMydeP-No	aaggaaaaaagcggccgCCTTAGGATATATCATGGGTAAAACAATCAG	*Not*I
pBMydeP-Ni	CGGATTTTaTgcaAGGTtgcGtCTAATAGCCTTCACAAAATAATTATCCGCGC	
pBMydeP-Ci	GCTATTAGaCgcaACCTtgcAtAAAATCCGCGCACAATTGAAGCC	
pBMydeP-Co	cgcacgcatgtcgaCCACGTTCCTGTAGAGGATTGATG	*Sal*I
ydeP-Wf	GAAGGCTATTAGCCTACACCTGTAAG	
ydeP-Mf	GAAGGCTATTAGaCgcaACCTtgcAt	
pBM1500-No	aaggaaaaaagcggccgCAATTTTGCGCTGGCAATGC	*Not*I
pBM1500-Ni	TaTgcaAGCTtgcGtCAGAAGTTTATATCTTATAGTATGTAGTTTAATTTAATCCATGTG	
pBM1500-Ci	AGATATAAACTTCTGaCgcaAGCTtgcAtAAACTCCGCTCAGTACTGAAGC	
pBM1500-Co	cgcacgcatgtcgacTACTGGCATAACCACATTGTTCGG	*Sal*I
b1500-Wf	GATATAAACTTCTGCCTACAGCTGTAAG	
b1500-Mf	GATATAAACTTCTGaCgcaAGCTtgcAt	
pRydePbla-No	aaggaaaaaagcggccgcAAACCTGGTACAAGGCTCAGC	*Not*I
pRydePbla-Ni	cggaaatgttgaatacTCATCTACTTATCCTGTGTGGAAATGAATTATTAGG	
pRydePbla-Ni2	ccacacaggataagtagATGAGTATTCAACATTTCCGTGTCGC	
pRydePbla-Ci2	gaagaaatgagaagaggcaTTACCAATGCTTAATCAGTGAGGCAC	
pRydePbla-Ci	ctcactgattaagcattggTAATGCCTCTTCTCATTTCTTCTGCTG	
pRydePbla-Co	cgcacgcatgtcgaCTTCAACAGGCTTTCACTGATGTACAG	*Sal*I
pRydeObla-No	aaggaaaaaagcggccgCTATAACCAGCGCATCCGTCATC	*Not*I
pRydeObla-Ni	cggaaatgttgaatactCATTTTATCTCCTTAAAACAATAAAGTTTTTATCGATAC	
pRydeObla-Ni2	ctttattgttttaaggagataaaATGAGTATTCAACATTTCCGTGTCGC	
pRydeObla-Ci2	gactactcgttagcaaataaTTACCAATGCTTAATCAGTGAGGCAC	
pRydeObla-Ci	ctcactgattaagcattggtaATTATTTGCTAACGAGTAGTCAACCACAC	
pRydeObla-Co	cgcacgcatgtcgaCGTTTGTTTATCTTCCGGTACCG	*Sal*I
ydeP	AAGGCTATTAGCCTACACCTGTAAGAAAATCCGCGC	
b1500	ATAAACTTCTGCCTACAGCTGTAAGAAACTCCGCTC	
yegR	TGTCCGTAATTCCTACTTATGTAGGAAATGTTGTAC	
evgA	GCAATACAATTCTTACGCCTGTAGGATTAGTAAGAA	
yfdW	CACCGGCGCTTCTTACAGTTGTAAGAATAACATCAC	
yfdX	GGAAGCATATTCCTACAATTGTAAGACTAAAATACT	
yqjI	TAAAAATCATT*T*TTACACTTGCAAGAACGCTCATAT	
ugpB	CACCTTACTATCTTACAAATGTAA*C*AAAAAGTTAT	
yhiU	GTGCAAGATTTCTTACGCATG*A*AAGAAGAGGTCGCC	

a. Sequences complementary to the amplified regions are represented with capital letters, and newly added sequences are represented with lowercase letters. Incorporated cutting sites for restriction enzyme (N, C, No and Co), start and stop codons of the gene or tandem group of the genes to be disrupted (Ni, and Ci for pKO plasmids), start and stop codons of bla (Ni, Ni2, Ci2 and Ci for pR plasmids) and consensus sequences (Ni, and Ci for pBM plasmids and DNA probes for gel mobility shift assay) are underlined. Substituted bases at highly conserved bases in yqjI, ugpB and yhiU are italicized.

b. Restriction enzyme for cutting amplified fragment.

reading frame. Sequence analysis confirmed the in frame insertion and the absence of PCR-generated mutations. The resulting plasmids express fusion proteins that have an additional 12 amino acids (Met-Arg-Gly-Ser-His-His-His-His-His-Gly-Ser) at the N-terminus and are under the transcriptional control of an IPTG-regulated phage T5 promoter. To make a constitutive EvgA expression plasmid, pHSGevgA, a *Hin*dIII–*Eco*RI fragment from pUCevgA was cloned into the *Hin*dIII–*Eco*RI site of pHSG396 (Takeshita *et al*., 1987).

Construction of plasmids for homologous recombination

Plasmids for the *hdeA–D* and *gadX* deletions, pKOhdeA–D and pKOgadX, respectively, plasmids for mutagenesis at EvgA binding sites upstream of *ydeP* and *b1500*, pBMydeP and pBM1500, respectively, and plasmids for replacement of *ydeP* and *ydeO* with *bla*, pRydePbla and pRydeObla, respectively, were constructed as follows. Approximately 1 kb fragments flanking the gene to be deleted or replaced or flanking

the 18 bp sequence to be mutated were amplified from genomic DNA of MG1655 or strains with EvgA binding site mutation in two separate PCRs using the No and Ni primer pairs or the Ci and Co primer pairs (Table 5). A full-length ORF of *bla* was also amplified from pBR322 using Ni2 and Ci2 primer pairs. The two or three PCR products were combined and amplified by PCR using primers No and Co. The resulting amplified products were digested with restriction enzymes and cloned into pKO3 (Link *et al*., 1997). The No and Co primers contain restriction enzyme cutting sites suitable for cloning. The Ni and Ci primers used to create deletions were designed such that the ends of the PCR products contained a sequence suitable for in frame fusion of the 5′ and 3′ flanking regions of the genes to be disrupted. These primers were designed to delete the *hdeA–hdeD* region leaving only the stop codon of each gene and to delete *gadX* leaving the stop codon and the first 24 nucleotides of the ORF. The Ni and Ci primers used to introduce binding site mutations contain the mutations. The Ni, Ni2, Ci2 and Ci primers used for gene replacements were designed such that the ends of the PCR products contained a sequence suitable for in frame fusion of *bla* and the flanking region of the gene to be replaced.

Allelic exchange and screening

Each plasmid for deletion, mutagenesis or gene replacement was electroporated into cells. After a 1 h recovery at 30°C with aeration, cells were plated on LB plates containing chloramphenicol and incubated at 43°C overnight. Individual integrants were colony purified on LB plates containing chloramphenicol and grown at 43°C for 12 h. Purified integrants were then grown in drug-free LB broth for 9 h to permit the second allelic exchange and then diluted, plated on LB plate supplemented with 10% (w/v) sucrose and incubated at 30°C for 24 h. Sucrose-resistant clones were selected and screened for chloramphenicol susceptibility. The verification of deletion and gene replacement was done by direct amplification of genomic DNA from each colony using a primer pair No and Co. A wild-type or deletion genotype was diagnosed according to the size of the PCR product. The verification of mutation was done by direct amplification of genomic DNA from each colony using primer pairs Wf and Co or Mf and Co. The Wf and Mf primers were designed for amplification of 1 kb fragment from the wild-type strain and mutant respectively. The presence of the correct binding site mutations was confirmed by sequence analysis. Using these methods, we constructed Δ*gadX*, Δ*acrB*Δ*ydeO*, Δ*acrB*Δ*hdeA–D*, Δ*acrB*Δ*hdeD-yhiE*, Δ*evgAS*Δ*ydeO*, Δ*yhiE*Δ*ydeP*, EvgA binding site mutants BMydeP, BM1500 and BMydeP1500 and six *bla*-introduced strains described in Fig. 3.

Acid resistance assay

A single colony of an *E. coli* strain harbouring plasmid was inoculated in 1 ml of LB broth containing carbenicillin and grown overnight with aeration at 37°C. LB broth (20 ml) was inoculated with 0.1 ml of the overnight culture and grown at 37°C with aeration. Expression of the His-tagged proteins was induced by adding IPTG to the LB broth before inoculation of the culture. When the cultures reached a cell density of 2×10^8 cfu ml^{-1}, 50 μl of the culture was transferred to 2 ml of phosphate-buffered saline (PBS; pH 7.2) and to 2 ml of warmed LB broth (pH 2.5, adjusted with HCl). The cfu ml^{-1} in PBS was determined by plating serial dilutions in PBS buffer (pH 7.2) on LB agar and using these as initial cell populations. The LB broth (pH 2.5) inoculated with *E. coli* was incubated at 37°C for 1 h, and the cfu ml^{-1} in LB broth (pH 2.5) was determined as described above and used as final cell populations. Percentage acid survival was then calculated as the number of cfu ml^{-1} remaining after the acid treatment divided by the initial cfu ml^{-1} at time zero. Each experiment was repeated two or three times. Percentage survival values were converted to logarithmic values ($\log_{10}$ x, where x equals the percentage survival) for the calculation of geometric means and standard errors (SE).

RNA preparation and microarray analysis

Total RNA was isolated from exponential phase cells by extraction with hot acid phenol–chloroform and purified with an RNeasy mini kit (Qiagen). Expression of the His-tagged proteins was induced by the addition of IPTG to LB broth, when required. Enrichment of mRNA, biotin labelling, hybridization and scanning were done as described in the *GeneChip Expression Analysis Technical Manual* (Affymetrix). Data analysis was performed using Affymetrix MICROARRAY suite 5.0 software. The software calculates change calls, change *P*-values (statistical significance for change calls) and signal log ratio. Arrays were scaled globally to a target signal of 500 and normalized. The default parameters for change calls were used. Signal log ratio is the change in expression level for a transcript between a baseline and an experiment array, expressed as the $\log_2$ ratio. All experiments were performed in duplicate or in two analogous conditions. Change in expression levels that had a change call of 'increase' or 'decrease' in both experiments was considered significant. The complete data set of microarray can be found at http://arep.med.harvard.edu/cgi-bin/ExpressDBecoli/EXDStart.

β-Lactamase reporter assay

Cells were grown at 37°C in LB broth containing chloramphenicol with aeration to an optical density at 600 nm of 0.2. Cells were harvested by centrifugation, suspended in ice-cold 50 mM potassium phosphate buffer (pH 7.0) and then broken by sonication. Cellular debris was removed by centrifugation. The enzyme activity in the supernatant was assayed spectrophotometrically at 386 nm with 100 μM nitrocephin (O'Callaghan *et al*., 1972) used as a substrate at 37°C. The activity in MG1655 was subtracted as background because MG1655 expressed very low levels of β-lactamase from the chromosomal *ampC* gene.

Purification of His-tagged EvgA

Cells were grown at 37°C in LB broth containing carbenicillin with aeration to an optical density at 600 nm of 0.5. IPTG was added at a final concentration of 1 mM to induce the expres-

sion of His-tagged EvgA, and the incubation was continued at 37°C for 4 h. Cells were harvested by centrifugation at 3000 *g* for 15 min, resuspended in 1 ml of lysis buffer (50 mM NaH_2PO_4, 0.3 M NaCl, 10 mM imidazole, 1 mg ml^{-1} lysozyme, pH 8.0), incubated on ice for 30 min and sonicated. Cellular debris was removed by centrifugation at 10 000 *g* for 25 min at 4°C. The supernatant (600 ml) was applied to a Ni-NTA spin column (Qiagen), which had previously been equilibrated with lysis buffer. Proteins were allowed to bind for 2 min at 4°C while centrifuging at 700 *g* and washed four times with 600 μl of wash buffer (50 mM NaH_2PO_4, 0.3 M NaCl, 20 mM imidazole, pH 8.0). His-tagged EvgA was eluted three times with 200 μl of elution buffer (50 mM NaH_2PO_4, 0.3 M NaCl, 250 mM imidazole, pH 8.0), and three fractions were collected. An aliquot of each fraction was mixed with gel loading buffer and subjected to SDS-PAGE. The second fraction contained highly purified EvgA protein and was used for gel mobility shift assay.

Gel mobility shift assays

DNA fragments (36 bp) were prepared by annealing two complementary oligonucleotides (Table 5). The 10 μl binding reactions contained 10 mM Hepes (pH 7.5), 50 mM KCl, 0.5 mM dithiothreitol (DTT), 0.1% IGEPAL CA-630 (Sigma-Aldrich), 10% glycerol, 4 ng of Cy3-labelled DNA probe and 200 ng of purified EvgA. Where indicated, 200 ng of competitive DNA was added to the reaction mixture. Binding was allowed to reach equilibrium at 37°C for 30 min, and then loaded onto a 6% polyacrylamide-0.5× Tris borate–EDTA native gel (Novex DNA retardation gel; Invitrogen). Samples were separated at 100 V and 4°C. The free DNA and DNA–protein complex bands were visualized using a Molecular Imager FX system (Bio-Rad).

Susceptibility testing

The antibacterial activities of the agents were determined by the broth microdilution method by following the recommendations of the National Committee for Clinical Laboratory Standards (1997) except that LB broth was used instead of cation-adjusted Mueller–Hinton broth, and the initial concentrations of some compounds differed from those recommended. The expression of His-tagged proteins was induced by adding 1 mM IPTG to the LB broth before inoculation of cells, when required. The lowest concentration of agent that completely inhibited growth was identified as the minimum inhibitory concentration (MIC). All agents used for susceptibility testing were obtained from Sigma-Aldrich.

Acknowledgements

We thank Wayne Rindone for construction of the microarray database. We thank Jay Shendure and Kazuyuki Yanai for help with ALIGNACE analysis and gel mobility shift assays respectively. We thank Daniel H. Levin and Aimee M. Dudley for careful reading of the manuscript. This work was supported by grants from the Department of Energy and the National Science Foundation to G.M.C.

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