GadE (YhiE): a novel activator involved in the response to acid environment in *Escherichia coli*

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In several Gram-positive and Gram-negative bacteria glutamate decarboxylases play an important role in the maintenance of cellular homeostasis in acid environments. Here, new insight is brought to the regulation of the acid response in *Escherichia coli*. Overexpression of yhiE, similarly to overexpression of gadX, a known regulator of glutamate decarboxylase expression, leads to increased resistance of *E. coli* strains under high acid conditions, suggesting that YhiE is a regulator of gene expression in the acid response. Target genes of both YhiE (renamed GadE) and GadX were identified by a transcriptomic approach. *In vitro* experiments with GadE purified protein provided evidence that this regulator binds to the promoter region of these target genes.

Several of them are clustered together on the chromosome and this chromosomal organization is conserved in many *E. coli* strains. Detailed structural (*in silico*) analysis of this chromosomal region suggests that the promoters of the corresponding genes are preferentially denatured. These results, along with the G + C signature of the chromosomal region, support the existence of a fitness island for acid adaptation on the *E. coli* chromosome.

**INTRODUCTION**

Enteric bacteria have developed mechanisms to maintain pH homeostasis under low pH conditions. These include extreme gastric acidity and volatile fatty acids produced by fermentation in the intestine, within the phagolysosomes of intestinal epithelial cells or in neutrophilic macrophages (Foster & Moreno, 1999). Indeed, intracellular pH is tightly regulated because of its global (Dilworth & Glenn, 1999) and specific effects on protein stability and biochemical reactions. First, to maintain pH homeostasis under mild acid shock, bacteria induce a change in the composition of outer-membrane proteins and in cell-surface hydrophobicity (Dilworth & Glenn, 1999). Next or concomitantly, Gram-negative and Gram-positive bacteria induce mechanisms specifically associated with pH resistance such as those involved in the synthesis of degradative amino acid decarboxylases (Auger *et al*., 1989; Castanie-Cornet *et al*., 1999; Gale, 1946; Rescei & Snell, 1972; Tabor & Tabor, 1985).

Decarboxylase activity resulting from lysine, arginine and glutamate decarboxylases were proposed to enhance growth under acid conditions by neutralizing the medium (Dilworth & Glenn, 1999; Small & Waterman, 1998). Whereas lysine decarboxylase and arginine decarboxylase systems are widely present in enterobacteria, the glutamate decarboxylase system is present in different Gram-positive and Gram-negative bacteria living in a similar ecological niche but which have no evolutionary link such as *Clostridium perfringens*, *Shigella flexneri* or *Escherichia coli* (Small & Waterman, 1998). This system consists of three genes, i.e. *gadA* and *gadB* encoding highly homologous glutamate decarboxylase isoforms and *gadC* encoding a putative glutamate:GABA antiporter. The *gadB* and *gadC* genes form an operon and a number of factors, including H-NS, RpoS and CRP-cAMP, are involved in the transcriptional control of *gad* expression (Castanie-Cornet & Foster, 2001; De Biase *et al*., 1999; Rowbury, 1997; Rowland *et al*., 1984).

Using genome-wide technologies such as transcriptome analysis (Velculescu *et al*., 1997), GadX was identified as a regulator of the glutamate decarboxylase genes from the AraC family in *E. coli* (Hommais *et al*., 2001; Ma *et al*., 2002). As for ToxR from *Vibrio cholerae*, which is an acid-induced factor and a virulence regulator, GadX has been shown to be involved in the appropriate expression of genes required for virulence of enteropathogenic *E. coli* (Shin *et al*., 2001).

In the present work we present evidence that YhiE, renamed GadE, is a regulator involved in the regulation of several...
genes required in the maintenance of pH homeostasis. Several of them are grouped in a specific region of the E. coli chromosome and may constitute an ecological fitness island.

METHODS

Bacterial strains, plasmids and growth conditions. Strains and plasmids used in this study are listed in Table 1. To overexpress yhiE, its coding sequence was amplified by PCR from genomic DNA using primers 5’-AGAGTCCGTGAAGCTTGATGC-3’ and 5’-ATCTGGATCCCTGATCGC-3’. These primers introduced a BamHI cloning site at the 3’ end and a HindIII site at the 5’ end. The PCR product was inserted into the HindIII and BamHI sites of pSU18 (Bartholomé et al., 1991), giving rise to plasmid pDIA578. pDIA590 is pDIA561 (Hommais et al., 2001) containing a cloned DNA fragment corresponding to the region located between 3 658 937 and 3 663 937 bp of E. coli (Hommais et al., 2001). It was selected for its ability to confer an increased resistance to the wild-type strain at low pH. Bacteria were grown at 37°C in M9 medium (Miller, 1992), pH 5.5 and 7, supplemented with 0.4% glucose and 0.012% glutamate. Selective antibiotics were added as needed at the following concentrations: chloramphenicol, 20 μg ml⁻¹; ampicillin, 100 μg ml⁻¹. All experiments were performed in accordance with the European regulation requirements concerning the contained use of genetically modified organisms of Group I (agreement no. 2735).

Low pH resistance. Bacteria were grown overnight in M9 medium (Miller, 1992), pH 5.5, supplemented with 0.4% glucose and 0.1% Casamino acids. Cells were diluted 1:1000 in M9 medium, pH 2.5, supplemented with 0.2% glucose and 0.012% glutamate (Lin et al., 1995) for 2 h at 37°C and then plated on LB. Viable cells were counted after 16 h at 37°C.

Macroarrays. Amplification reactions were performed in 96-well plates (Perkin Elmer) in a 100 μl reaction volume containing 10 ng chromosomal DNA of E. coli, 3 mM MgCl₂, 0.2 mM dNTPs (Perkin Elmer), 0.3 mM each primer (E. coli ORF mer; Sigma Genosys) and 2·6 U Expand High Fidelity (Roche). Reactions were cycled 30 times (94°C, 15 s; 55°C, 30 s; 68°C, 3 min) with a final cycle of 72°C for 10 min in a thermocycler. Normalization was performed with a foreign gene encoding the luciferase of Photinus pyralis, which was amplified and printed on nylon membranes 15 times. Amplification of each PCR product was verified on agarose gels. For array preparation, nylon membranes (Qfilter; Genetix) were soaked in 10 mM Tris/1 mM EDTA (TE), pH 7.6. The ORF-specific PCR products and controls were printed using a Qpix robot (Genetix). Immediately following spot deposition, membranes were denatured and neutralized for 15 min in 0·5 M NaOH/1·5 M NaCl, then washed three times with distilled water and stored wet at −20°C until use.

RNA preparation. After growth and centrifugation of bacterial cells as described previously (Hommais et al., 2001), cells were lysed and RNA was extracted by a rapid procedure using FastPrep FP120 (Bio-101) and trizol solution (Invitrogen). RNA was redissolved in 100 μl TE. To remove genomic DNA, RNA was incubated for 15 min at 37°C with DNA-free DNase from Ambion, according to the manufacturer’s recommendations, and quantified by measuring A260 and A380. RNA purity and integrity were controlled by separating a sample on an agarose gel and ensuring that mRNA, tRNA and rRNAs could be seen.

cDNA probe synthesis. Hybridization probes were generated from 1 μg RNA and 0.5 ng luciferase mRNA from Photinus pyralis (Invitrogen) by incubating AMV reverse transcriptase (Roche) for 2 h at 42°C following a standard cDNA synthesis procedure using [α-32P]dCTP [2000–3000 Ci mmol⁻¹ (74–111 TBq mmol⁻¹); New England Nucleic] as described previously (Hommais et al., 2001); 0.5 ng luciferase-purified mRNA was added before reverse transcription for the normalization procedure. Unincorporated nucleotides were removed from labelled cDNA by gel filtration through a G-25 Sephadex column (Roche). Pre-hybridization and hybridization were carried out as described previously (Hommais et al., 2001).

Data analysis. Analysis of the data was performed as described previously with some modification (Hommais et al., 2001). Exposed PhosphorImager screens were scanned on a 445Si PhosphorImager (Molecular Dynamics) with a pixel size of 177 μm. The intensity of each dot on the resulting TIFF image files was measured with XDOSREADER software (Cose) and analysed using an Excel spreadsheet. The background noise was calculated from the intensity around each dot. Dot intensity was normalized according to the mean value of the intensities of luciferase spots on each DNA array, which allowed direct comparison of the two strains.

Primer extension. Primer extensions were performed as described previously (Soutourina et al., 1999) with the following modification. The transcription start site of gadE was determined with 10 μg RNA from BE1410 containing pDIA578 and cultivated at pH 5.5 or 7. Analysis of the hdeD gene was performed with 20 μg RNA from a strain that expressed hdeD at a high level (BE1410 containing pDIA590) cultivated at pH 7. Direct sequencing on chromosomal DNA was performed as described previously (Krini et al., 2001).

Protein purification. To overexpress the GadE recombinant protein, its coding sequence was amplified by PCR from genomic DNA using primers 5’-GGAGATTCCATATGATTTTCTCATGACGAA-3’.
and 5′-CCGCCGGAAAATAAGATGTGATACCC-3′. These primers introduced an NdeI cloning site at the 3′ end and an Xmal site at the 5′ end. The PCR product was inserted into the NdeI and Xmal sites of the plexiv 2.3 MCS plasmid (Roche) giving rise to pDIA579. This introduced six histidine residues at the end of the GadE protein, which was purified as described previously (Bertin et al., 1999).

**Gel mobility shift assay.** Oligonucleotides used in this study are listed in Table 2. DNA fragments were amplified by PCR using the DyNAzyme EXT kit (Finnzymes) after labelling one end with [γ-32P]ATP and T4 polynucleotide kinase. PCR fragments were purified using the QIAquick PCR purification kit (Qiagen). For gel mobility shift assays, GadE was bound to the labelled DNA fragment (30 ng) in 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 10% glycerol, 400 μg BSA ml⁻¹, 80 mM NaCl, 100 μg poly-dIdC ml⁻¹ and 20 mM glutamate at room temperature for 30 min. The gel shift was visualized, after migration in a 6% acrylamide gel in 0.5-5 x TBE buffer and 20 mM glutamate, with a PhosphorImager (Molecular Dynamics).

**RESULTS**

**Identification and characterization of genes involved in the acid stress response**

We have previously investigated the regulation underlying glutamate-dependent acid resistance and demonstrated a positive role for GadX in this process (Hommais et al., 2001). Its corresponding gene was isolated by overexpression of E. coli genes from a genomic DNA library. A second clone, carrying plasmid pDIA577 (Table 1), resistant to low pH was obtained by a similar procedure. This plasmid also conferred an increased glutamate-dependent acid resistance to the wild-type strain (25% of bacterial cells survived exposure to acid stress whereas only 0.01% of bacterial cells from wild-type strain FB8 survived exposure to the same stress). Sequence determination allowed us to identify the cloned DNA fragment, which corresponds to the region located between 3655850 and 3656897 bp on the E. coli K-12 chromosome carrying the full coding sequence of yhiE flanked by intergenic regions. This gene, previously shown to be induced in the hns mutant strain (Hommais et al., 2001), encodes a potential regulator of the LuxR/AhpC family similar to YhiF of E. coli (Tucker et al., 2002).

Prediction of the secondary structure of YhiE suggests the existence of a potential helix–turn–helix DNA-binding domain between amino acids 131 and 152 (data not shown), which further supports a regulatory function for YhiE. To determine whether YhiE could enhance acid resistance, its structural gene was amplified by PCR, cloned and overexpressed from plasmid pDIA578 in an E. coli wild-type strain (Table 1). Remarkably, 18% of bacterial cells survived exposure to acid stress, whereas only 0.01% of bacterial cells

| Table 2. Oligonucleotides for gel mobility shift assays |
|-------------------------------------|-----------------|-----------------|
| Primers   | Sequence (5′-3′) | Reference       | Size of fragment (bp) |
| gadX-1    | CGCAATAATATATTGCTGT | This study     | 325            |
| gadX-2    | ATGTAGTGATGTAGTTGTG | This study     | 325            |
| gadE-1    | GTGAAACAAGGAGACTCGA | This study     | 285            |
| gadE-2    | TGTTCAATATAGTAAACGCC | This study    | 285            |
| osmC-1    | GCTTGTGTGGTTAGATTAGT | This study    | 263            |
| osmC-2    | CATTTGCTTCTCCTCGTGGG | This study    | 263            |
| osmC-3    | GTGGGATTATTTATTAAGTGC | This study   | 127             |
| ompC-1    | ATCCGGACTTTCATGTATTA | This study    | 265            |
| ompC-2    | CCTGAAATTTATGGCTTG | This study    | 265            |
| rcsA-1    | ATTTTGTCTGTAGATATAT | This study    | 231            |
| rcsA-2    | TTGTTTACGGCCGCTTACTA | This study    | 231            |
| rfaQ-1    | TATGACCGAGGTCTTTCGA | This study    | 337            |
| rfaQ-2    | ATCTGCGCTACATCTTCAT | This study    | 337            |
| gltB-1    | CAAAATACCGGAAATTTCAT | This study    | 435            |
| gltB-2    | TCCGATCGGTTTAATACGTT | This study    | 435            |
| gadA-203  | GAACCTCCTAAAATTTTGG | This study    | 325            |
| gadA-201  | TTGGGCGAGTTTATTACG | Castanie-Cornet & Foster (2001) | 185   |
| hde-1     | CGACACTGATTGATTAAACCTGG | This study | 162          |
| hde-2     | ATGCCCCAAACCCGTCTAAG | This study    | 162            |
| gadX-2    | ATGTAGTGATGTAGTTGTG | This study    | 137            |
| gadX-3    | CATCACACATTATCATCTGCTTCTCCTCCGCTT | This study | 137       |
| gadA-203  | GAACCTCCTTAAATTATTTTG | This study    | 137            |
| gadA-261  | GTGGTTTTTCTGCTTAG | Castanie-Cornet & Foster (2001) | 120   |
| gadE-2    | TGTTCAATATAGTAAACGCC | This study    | 124            |
| gadE-3    | AGGAATCTTACTTATAGGTACCA | This study | 124            |
from the wild-type strain (FB8) survived exposure to the same level of stress, demonstrating that sole overexpression of YhiE increased acid resistance.

To further characterize the yhiE regulatory gene we determined its transcription start site by primer extension experiments with total RNA from hns strains cultivated at pH 7 and 5·5 with pDIA578 (Table 1, Fig. 1). Two transcription start sites located 92 and 125 nt, respectively, upstream from the ATG start codon were identified (Fig. 1), whereas a unique site was observed in the gadX regulatory region (Tramonti et al., 2002). A similar result was obtained from wild-type and hns strains without pDIA578 (data not shown). A strong increase in the level of these two transcripts was observed when RNA was extracted from cells cultured at low pH (Fig. 1) and in an hns genetic background. This is in agreement with previous transcriptome analysis of an hns mutant and under acidic conditions where transcription of yhiE and gadX were shown to be induced (Hommais et al., 2001; Tucker et al., 2002). Taken together, this strongly suggests that YhiE is involved in bacterial resistance to low pH and the protein was therefore renamed GadE.

**Effect of GadX and GadE on expression profiling at low pH**

To determine the role of GadE or GadX in bacterial acid resistance we used a transcriptomic approach. Both gadX and gadE genes have been shown previously to be regulated by H-NS (Hommais et al., 2001). This suggests that the targets of the corresponding proteins are also indirectly regulated by H-NS. To identify them we elaborated macroarrays containing DNA fragments corresponding to the 250 coding genes of the H-NS regulon (see Methods). RNAs were isolated from the wild-type strain with or without pDIA570 or pDIA578 and cultivated at pH 7. cDNAs were prepared and hybridized as described previously (Hommais et al., 2001). To account for unspecific variations, experiments were carried out using at least three independent RNA preparations from which at least two hybridizations were performed from two different sets of DNA arrays.

Comparison of the signal intensity of arrays from duplicates or from independent hybridizations showed a high reproducibility of the results. Comparison of gene expression between cells cultivated at neutral pH using the non-parametric statistical test of Wilcoxon did not allow us to identify genes significantly regulated by the overexpression of gadE or gadX. Similar results were recently obtained in gene expression analysis of a gadX mutant strain (Tucker et al., 2003). As cells were adapted by growth overnight at pH 5·5 before the acid resistance assay (see Methods), we analysed the expression profile of strains under conditions that increased acid resistance: overexpressing gadE or gadX under neutral and acid conditions during exponential-phase cell growth. Comparison of gene expression between cells cultivated at low pH allowed us to identify genes specifically induced by the overexpression of gadE or gadX at pH 5·5 (Table 3). This suggests that overexpression of either GadX or GadE alone, in an FB8 genetic background, is not sufficient to induce expression of genes involved in the bacterial response to low pH when cells are grown at neutral pH.

Overexpression of gadX significantly induced the expression of 48 genes of the H-NS regulon (Table 3a). Several of them are known to be involved in the bacterial acid response, such as the genes of the glutamate decarboxylase system (gadA, gadB and gadC), the lysine decarboxylase system (cadA, cadB), the regulator-encoding gene (gadW/yhiW) or other acid-induced genes of unknown function (hideA, hideB and hdeo). Most of these genes were induced by overexpression of gadX in bacterial cells cultivated at pH 5·5. The results obtained were essentially the same as those of Tucker et al. (2003), except for gadW, which is induced by the overexpression of gadX. This could be due to a difference in the experimental procedures used in the two experiments: the strains and conditions of culture were indeed not similar. Remarkably, gadE was induced when gadX was overexpressed. Moreover, genes known to be involved in bacterial adaptation to detrimental growth conditions, such as high osmolarity (osmC), were also induced by gadX overexpression, as well as genes encoding proteases or chaperones (lon, ycgG, yehA and yhcA). Interestingly, a recent study has shown that a Salmonella typhimurium strain deficient for the Lon protease shows an increased sensitivity to acid stress while its virulence is attenuated (Takaya et al., 2003). Three genes spotted on the arrays and involved in glutamate biosynthesis were also induced by gadX...
Table 3. GadE and/or GadX target genes

(a) Gene* | Protein function | P value | Expression log ratio between strain overexpressing GadX vs WT at pH 5.5
--- | --- | --- | ---
appB | Cytochrome oxidase | 0.0367 | 0.33
asnB | Asparagine synthetase | 0.0125 | 0.27
cadA | Lysine decarboxylase | 0.0284 | 0.27
cadB | Lysine/cadaverin antiporter | 0.0125 | 0.24
cspC | Multicopy suppresses mukB mutants | 0.0051 | 0.28
fiIA | RNA polymerase suppresses flagellar operon | 0.0469 | 0.48
gadA | Glutamate decarboxylase | 0.0051 | 0.67
gadB | Glutamate decarboxylase | 0.0051 | 0.51
gadC | Glutamate/GABA antiporter | 0.0051 | 0.54
gadE | Unknown function | 0.0093 | 0.27
gadW | Similar to AraC regulator family | 0.0051 | 0.5
gadX | Similar to AraC regulator family | 0.0051 | 0.7
glnH | Periplasmic glutamine-binding protein | 0.0469 | 0.28
glnK | Regulator protein | 0.0469 | 0.37
gspE | General secretory pathway genes | 0.0367 | 0.24
hdeA | Periplasmic, unknown function | 0.0051 | 0.52
hdeB | Periplasmic, unknown function | 0.0069 | 0.3
hdeD | Periplasmic, unknown function | 0.0469 | 0.34
hha | Histone-like; downregulates gene expression | 0.0284 | 0.13
hns | DNA-binding protein | 0.0051 | 0.23
lon | Protease | 0.0284 | 0.27
modF | Molybdate uptake | 0.0367 | 0.22
osmA | Unknown function, induced by high osmolarity | 0.0166 | 0.28
rnhB | RNase HII | 0.0367 | 0.28
rpoE | RNA polymerase, sigma E-subunit | 0.0166 | 0.28
rpoS | RNA polymerase, sigma S-subunit | 0.0051 | 0.18
sfbB | 23S rRNA pseudouridine synthase | 0.0051 | 0.2
sgcA | Putative phosphotransferase IIA | 0.0469 | 0.34
sirA | Small protein required for cell growth | 0.0284 | 0.2
xapR | Regulator for xanthosine phosphorylase | 0.0284 | 0.21
ybaS | Putative glutaminase | 0.0469 | 0.24
ycgG | Putative protease | 0.0367 | 0.23
ydeO | Similar to AraC regulator family | 0.0125 | 0.3
ydgK | Unknown function | 0.0367 | 0.23
ydgL | Putative membrane protein | 0.0218 | 0.28
yegZ | Unknown function | 0.0069 | 0.2
yehA | Putative type-1 fimbrial protein | 0.0051 | 0.3
yffL | Unknown function | 0.0166 | 0.21
yfdH | Putative glycogen biosynthesis enzyme | 0.0166 | 0.23
ygaP | Unknown function | 0.0367 | 0.22
ygcK | Unknown function | 0.0469 | 0.27
ygcL | Unknown function | 0.0093 | 0.25
yhcA | Putative chaperone protein | 0.0069 | 0.25
yjiX | Unknown function | 0.0069 | 0.25
yliA | Unknown function | 0.0051 | 0.22
ymcE | Putative receptor | 0.0469 | 0.26
yniA | Unknown function | 0.0218 | 0.31
yggD | Unknown function | 0.0367 | 0.37

(b) Gene* | Protein function | P value | Expression log ratio between strain overexpressing GadE vs WT at pH 5.5
--- | --- | --- | ---
cadB | Lysine/cadaverin antiporter | 0.0166 | 0.40
cspC | Multicopy suppresses mukB mutants | 0.0469 | 0.3
overexpression, i.e. asnB that encodes a structural protein involved in biosynthesis of asparagine that synthesizes glutamate as well, glnH that encodes a glutamine-binding protein and glnK that encodes the regulatory protein of the gln operon.

At low pH, gadE overexpression significantly induced genes that are involved in the bacterial acid response, such as gadA, hdeA, hdeD or gadX (Table 3b). Again, genes induced by the response to other stresses or encoding chaperones and proteases were also induced by gadE induction, e.g. osmC, hdeA and ycgG. Several genes involved in the biosynthesis of glutamate were induced by the overexpression of gadE, such as gltD and glnH. Strikingly, several genes involved in the biosynthesis of bacterial membrane components were also induced by gadE overexpression, for example rcsA that encodes the activator of colanic acid synthesis, and rfaG, a representative of the LPS core biosynthesis genes on our macroarrays. Moreover, several genes regulated by GadE encode proteins of unknown function. Similarity searches demonstrate that two of them are similar to type 1 fimbrial proteins YcbQ and YehA, and one of them is a potential membrane protein, YdgL.

DNA-binding properties of GadE

As mentioned in Table 3, the overexpression of gadE significantly induced the expression of genes that are

Table 3. cont.

<table>
<thead>
<tr>
<th>(b) Gene</th>
<th>Protein function</th>
<th>P value</th>
<th>Expression log ratio between strain overexpressing GadE vs WT at pH 5.5</th>
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<td>cspG</td>
<td>Cold-induced CspA/B analogue</td>
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<td>yliA</td>
<td>Unknown function</td>
<td>0.0218</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*Gene names in bold type are genes induced by both GadE and GadX.
involved in (i) the bacterial acid response (gadA, gadX), (ii) other stress responses or that encode chaperones (hdeA, osmC), (iii) the biosynthesis of bacterial membranes (ompC, rcsA, rfaG) and (iv) the biosynthesis of glutamate (gltD). To determine the impact of GadE in such pathways, we analysed the DNA-binding properties of GadE to the promoter region of several gene targets whose products are involved in each pathway. The regulatory region of some of them has been described in detail previously: gadA (Castanie-Cornet & Foster, 2001; De Biase et al., 1999), gadX (Tramonti et al., 2002), hdeAB (Arnvist et al., 1994; Tucker et al., 2003), gltBD (Wiese et al., 1997), osmC (Bouvier et al., 1998), rcsA (Stout, 1996), rfaGQPSBIJYZK (Clementz, 1992) and ompC (Norioka et al., 1986). We characterized here the promoter region of hdeD. A single transcriptional start site located 35 nt upstream from the ATG start codon was identified as an A (Fig. 2).

GadE gel mobility shift assays were first performed with DNA fragments ranging from 162 bp for hde to 435 bp for gltBD (Table 2), which corresponds to the whole intergenic region between the target genes and genes located immediately upstream. GadE was shown to bind to those fragments (Table 2, Fig. 3) and the binding capacity of this protein was increased in the presence of glutamate. This demonstrates that GadE binds promoter regions of gadA, gadX, hdeD, which is involved in acid resistance, hdeAB, which encode chaperones, ompC, rcsA and rfaQ, which are involved in biosynthesis of the bacterial membrane, and gltBD, which is involved in the biosynthesis of glutamate. Moreover, GadE was able to bind to a fragment of the gadE promoter (Table 2), suggesting that this gene is autoregulated (Fig. 3). Gel mobility shift assays were then performed with DNA fragments of 100 bp, corresponding to the promoter regions of some target genes: gadA (−75 to +24), gadE (−103 to +20), gadX (−90 to +46) and osmC (−105 to the ATG start codon), but no binding of GadE could be identified. This suggests that the GadE-binding site is located far away from the transcription start site, e.g. between −150 and −75 upstream from the ATG start codon for gadA, between −264 and −103 for gadE, between −278 and −90 for gadX and between −241 and −105 for osmC. Taken together, these results demonstrate that GadE directly controls the expression of at least these nine genes whose products are involved in acid resistance, membrane biosynthesis and glutamate biosynthesis.

**Chromosomal localization of genes involved in the low pH response**

Many genes identified by DNA array analysis (Table 3) are regulated by both GadX and GadE. Remarkably, several of these targets were previously shown to be greatly induced under acid conditions (Tucker et al., 2002) and located in the same region on the E. coli genome, i.e. between 3650 and 3665 bp. This DNA region contains nine genes, hdeB, hdeA, hdeD, gadE, yhiU, yhiV, gadW, gadX and gadA. Here we have demonstrated that GadE and/or GadX regulators control the expression of seven of them.
To characterize the regulatory region of these genes we analysed their DNA denaturation properties in silico (Yeramian, 2000; Yeramian & Jones, 2003). Remarkably, the promoter of the genes in this genomic region showed high propensities for helix disruption on DNA stability maps (plotting the probability of helix-opening along the sequence) (Fig. 4). By comparison, the non-coding DNA in the genomic regions adjacent to the acid cluster did not display such propensities for helix disruption with significantly higher melting temperatures for the corresponding promoters (Fig. 4). Based on the structural properties of promoters of genes from the acid cluster, these results

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**Fig. 4.** Helix-opening probability curves, corresponding to increasing temperatures, plotted superimposed with the sequence. The temperatures used are indicated on the figure (for example, T66 indicates holding for a temperature of 67 °C). All calculations followed methods and parameters described in Yeramian (2000) (see also Yeramian & Jones, 2003). Following the x-axis, the origin is set to 3 600 000 bp. The genetic annotation of the corresponding region (from bp 3 635 000 to 3 685 000) is represented above the probability curves (each gene being associated with an arrow).
sugest that these genes could be easily depressed, which may suggest the presence of an efficient locking mechanism to prevent untimely induction of these genes.

**Genomic comparison and G+C content**

To know whether this 15 kb acid cluster (hdeB-gadA) is conserved in other strains, the nucleotide sequence of this cluster was compared to *E. coli* strains whose genomic sequences are already known. Remarkably, this genomic organization is conserved in *E. coli* strains that are causative agents of various intra-intestinal diseases, such as the enterohaemorrhagic *E. coli* (EHEC) strain O157: H7 (EDL933), the enteropathogenic *E. coli* (EPEC) strain 2369 and extra-intestinal strains such as the uropathogenic *E. coli* (UPEC) strain CFT073 and the neonatal meningitis *E. coli* K1 strain RS218. The G+C content of each cluster was calculated and compared to that of the corresponding *E. coli* complete genome. In *E. coli* K-12 the nucleotide composition of this region showed a 46 mol% G+C content, whereas the nucleotide composition of the whole *E. coli* K-12 chromosome is 50–79 mol% G+C. Statistical analysis showed that the G+C content of the acid cluster is significantly lower when compared to the G+C content of the *E. coli* total genome (*P* < 0.001). Similar results were obtained in the different pathogenic *E. coli* strains, i.e. EDL933, CFT073, RS218 or 2369, suggesting that in each strain, the nucleotide composition of this region is different compared to the whole genome.

**DISCUSSION**

In accordance with our previous study (Hommais et al., 2001), overexpression of gadX or gadE (formerly called yhiE) allowed bacteria to increase their glutamate-dependent acid resistance. Amino acid sequence comparison suggests that the product of *gadE* (yhiE) is similar to YhiF, a putative regulatory protein of the LysR family. Like YhiF, GadE (YhiE) has a potential helix–turn–helix DNA-binding motif. Interestingly, comparison of *gadE* (yhiE) with all sequences present in databases showed that no similar protein could be found in other bacteria except *E. coli* and *Shigella* strains, suggesting that this gene is specific to these species. Recently *gadE* (yhiE) has been identified as inducible by low pH, its inactivation being detrimental to acid resistance in minimal medium (Tucker et al., 2002), or to acid resistance caused by EvgA overexpression (Masuda & Church, 2002, 2003), and *gadE* has been proposed to be regulated by YdeO (Masuda & Church, 2003). By primer extension experiments we identified two transcriptional start sites. Such a complex promoter structure suggests possible multiple regulation of this gene even though no different rate of transcription was identified between neutral and low pH and between wild-type and *hns* mutant strains, suggesting the presence of another regulation factor.

GadE seems to play an essential role in the bacterial acid response and is an activator of acid resistance, as is the case for GadX. Previous studies have demonstrated that the transcription of both *gadX* and *gadE* are downregulated by H-NS, suggesting that their targets are both components of the H-NS regulon (Hommais et al., 2001). To elucidate the mechanism underlying this higher resistance to acid stress via GadX and/or GadE, we analysed the effect of their overexpression on gene expression focusing our attention on H-NS gene targets. This led us to elaborate DNA macroarrays containing more than 200 genes of the H-NS regulon. First, no modification of gene expression could be detected at neutral pH with the overexpression of either GadX or GadE. Consistent with this, no significant difference in gene expression at neutral pH has been observed previously in a gadX mutant strain (Tucker et al., 2003). However, even if we could not rule out some false positives, our results showed that several genes could be identified as significantly induced at low pH, demonstrating that GadE and GadX act as transcriptional activators of genes of the H-NS regulon under acidic conditions. This could argue that both regulators have a role in cell adaptation to low pH. Under low pH conditions 30 and 19 genes were induced by overexpression of *gadX* and *gadE*, respectively, and 18 genes were induced by both regulators (GadX and GadE). In addition, gel mobility shift assays demonstrated that GadE could be autoregulated, and regulated GadX, suggesting that an indirect effect of GadE on the transcriptional control of some of the target genes identified here could not be ruled out.

A low pH environment leads to cytoplasm acidification in micro-organisms. This effect induces damages in macromolecules and results in growth arrest and bacterial lysis. To alleviate such effects and maintain pH homeostasis, bacteria have developed several mechanisms that can be divided into four steps: step 1, a cellular envelope modification to diminish ionic permeability (Benjamin & Datta, 1995; Dilworth & Glenn, 1999); step 2, the induction of DNA repair machinery and chaperones (Bearson et al., 1997) which results in major changes in gene expression (Dilworth & Glenn, 1999); step 3, the development of ionic pumping systems and proton extrusion/uptake (Dilworth & Glenn, 1999); and step 4, an indirect increase in external pH (Booth, 1985; Dilworth & Glenn, 1999; Small & Waterman, 1998). By performing functional clustering analysis on GadX and GadE regulons, we have presented evidence that both GadX and GadE are activators of the global bacterial acid response involved in the general adaptation of pH homeostasis maintenance in *E. coli*. Indeed, the targets of these genes are involved in three of the four steps of the bacterial response to cytoplasm acidification. In addition, transcriptional analysis of *gadE* overexpression at low pH demonstrated the induction of more than 10 genes whose products are involved in the biosynthesis of the bacterial envelope. These various features demonstrate that GadE is involved in the activation of this first bacterial response to acid stress. Analysis of the binding properties of GadE to double-stranded DNA demonstrated a direct binding of this protein to the promoter regions of *ompC*, *rfaQ* and *rcsA*, suggesting that GadE plays a direct role in the transcription of these...
Overexpression of both gadE and gadX induced the expression of several genes involved in the second step of the bacterial response to cytoplasmic acidification: the induction of DNA repair machinery and chaperones (Bearson et al., 1997). For example, hdeA was induced by overexpression of both gadE and gadX. This gene, along with hdeB and hdeD, was first identified as H-NS-repressed and RpoS-dependent and subsequently demonstrated to be induced by acid. Its product is a periplasmic chaperone that is presumably important for counteracting the deleterious effects of acid on periplasmic proteins by suppressing aggregation of proteins under acid conditions at pH 2. As hdeA and hdeB are transcribed together in a single operon (Arnqvist et al., 1994), it is possible to suppose that the function of HdeB is linked to that of HdeA. hdeD, which encodes an integral membrane protein of unknown function, is located immediately downstream of hdeAB and was also induced by overexpression of both gadX and gadE (Table 3). Gel shift experiments demonstrated that both GadX (Ma et al., 2002) and GadE bind to the promoter region of these genes, suggesting that they play a direct role in their activation. Moreover, several genes encoding putative chaperones and proteases were also induced by overexpression of gadX or gadE, e.g. ycgG and yhcA. In addition, overexpression of gadX induced the expression of several genes encoding regulatory proteins such as YdeO, a member of the AraC/XylS transcriptional regulator family. Deletion of the ydeO structural gene has been recently demonstrated to decrease acid resistance caused by EvgA overexpression, suggesting a function in the bacterial response to an acid environment (Masuda & Church, 2002, 2003).

Finally, the acid resistance response in the presence of glutamate has been previously shown to induce the glutamate decarboxylase system, which was suspected to catalyse the conversion of glutamate to α-amino butyrate and maintain a near to neutral intracellular pH when cells are exposed to extreme acid conditions. This corresponds to the fourth step of the E. coli response to cytoplasm acidification, i.e. the indirect external pH increase. GadX has been previously demonstrated to be involved in the transcriptional activation of the glutamate decarboxylase system (gadA/B, gadC) as a direct activator of these genes, which were up-regulated by overexpression of gadX (Hommais et al., 2001; Ma et al., 2002; Tramonti et al., 2002). These genes encode two glutamate decarboxylase isoenzymes and the putative glutamate antiporter. Gel shift assays demonstrated the binding of the GadX protein to gadA and gadW promoter regions, suggesting direct control of GadX in the regulation of these genes (Ma et al., 2002; Tramonti et al., 2002; Tucker et al., 2003). As for gadX, gadE is involved in the regulation of gadA. Our gel shift experiments showed a direct binding of this protein to the regulatory region of gadA, indicating that GadE may be an activator of this system. The existence of additional positive regulators involved in the transcriptional tuning of the gad system has been proposed previously (Tramonti et al., 2002). Our results strongly suggest that GadE is such an additional positive regulator. Remarkably, overexpression of gadX induced expression of glnH, glnK, asnB and ybaS, which encodes a putative glutaminase, although no direct binding to the promoter regions of these genes could be demonstrated (data not shown). Similarly, overexpression of gadE induced expression of glnH and gltD. This acid induction of genes involved in the formation of glutamate from glutamine points to a mechanism for the endogenous formation of glutamate in conjunction with glutamate-dependent acid resistance, which has been suspected (Tucker et al., 2002). Interestingly, GadE binds the gltBD promoter, suggesting a direct effect of this protein in the regulation of glutamate biosynthesis under acid conditions.

No consensus sequence for the binding of GadE on DNA could be identified. Therefore, the intergenic regions of the target genes were analysed in silico. Most promoter regions showed a high propensity to DNA double helix disruption, suggesting that these genes may be easily transcribed and need a very efficient locking mechanism to prevent any possible untimely induction of these genes under inappropriate conditions. Indeed, their transcription is regulated by at least three regulatory proteins: GadX, GadE and H-NS. This points out the complexity of the regulatory mechanism of the bacterial acid response, which makes E. coli highly adapted to situations that can lead to lethal acid stress. Moreover, as H-NS has been previously demonstrated to bind AT-rich DNA, which correlates well with a high DNA melting capacity, we hypothesize that the locking mechanism of acid genes is due to H-NS. Remarkably, seven of these genes, i.e. hdeB, hdeA, hdeD, gadE, gadW, gadX and gadA, are localized in the same E. coli chromosomal region between 3650 and 3665–2 kb. Most of them are induced by low pH (Tucker et al., 2002), suggesting that the presence of this cluster is needed for the survival of cells grown under these conditions. Moreover, the G + C content analysis of this region showed a significantly lower proportion than the mean G + C content of the total E. coli genome. Carriage of gene clusters with (i) specific functions, (ii) different G + C content in comparison to DNA of the host chromosome and (iii) distinct genetic units (capacity to disrupt DNA) are properties that define a ‘fitness island’ (Hacker & Carniel, 2001). Indeed, fitness islands can be considered as a component of ecological islands that enhance the adaptation of bacteria to survive or grow in their niches (Hacker & Carniel, 2001). Such an acid fitness island could be very useful to maintain pH homeostasis of bacteria in the stomach, in the intestine or within the phagolysosomes of epithelial cells. Remarkably, a genomic comparison of E. coli strains whose genomic DNA has been completely sequenced showed that the genomic organization of this island is highly conserved in E. coli pathogenic strains O157:H7, 2369, RS128 and CFT073, whereas this cluster could not be found.
in any other bacterial species. Although these comparisons require the analysis of more genomes to understand the origin of this island, our results suggest that despite a similar response to a low pH environment (Hommais et al., 2002), bacterial species have developed specific systems to regulate genes involved in pH homeostasis.

REFERENCES


