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Large-scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein, H-NS

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Summary

Despite many years of intense work investigating the function of nucleoid-associated proteins in prokaryotes, their role in bacterial physiology remains largely unknown. The two-dimensional protein patterns were compared and expression profiling was carried out on H-NS-deficient and wild-type strains of *Escherichia coli* K-12. The expression of approximately 5% of the genes and/or the accumulation of their protein was directly or indirectly altered in the *hns* mutant strain. About one-fifth of these genes encode proteins that are involved in transcription or translation and one-third are known to or were *in silico* predicted to encode cell envelope components or proteins that are usually involved in bacterial adaptation to changes in environmental conditions. The increased expression of several genes in the mutant resulted in a better ability of this strain to survive at low pH and high osmolarity than the wild-type strain. In particular, the putative regulator, YhiX, plays a central role in the H-NS control of genes required in the glutamate-dependent acid stress response. These results suggest that there is a strong relationship between the H-NS regulon and the maintenance of intracellular homeostasis.

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Introduction

Considerable progress has been made recently in genome analysis. The complete genome sequences of 35 microorganisms, including several pathogens, have been determined, and over 100 microorganisms are currently being sequenced (http://www.tigr.org/tdb/mdb/mdb.html). These studies have emphasized our lack of physiological understanding of living organisms, including model microorganisms, such as *Escherichia coli* (Blattner *et al*., 1997) and *Bacillus subtilis* (Kunst *et al*., 1997), which have been studied extensively. Indeed, the function of approximately 40% of the predicted genes has not been assigned (Blattner *et al*., 1997; Kunst *et al*., 1997). Moreover, many of the annotations assigned to genomes based on reference models are often either irrelevant or even spurious (Kyrpides and Ouzounis, 1999).

In enterobacteria, nucleoid-associated proteins are required for the organization of chromosomal DNA. These include HU, IHF, FIS and H-NS, which are small, abundant proteins (Talukder *et al*., 1999), as are several eukaryotic DNA-binding proteins (Hayat and Mancarella, 1995). H-NS was initially described as a transcription factor (Jacquet *et al*., 1971) and was later shown to play a role in the structure and functioning of chromosomal DNA (Atlung and Ingmer, 1997; Williams and Rimsky, 1997). In *E. coli*, this $\approx$15 kDa protein affects DNA supercoiling (Tupper *et al*., 1994) and condenses DNA (Dame *et al*., 2000) by preferentially binding to curved DNA *in vitro* (Jordi *et al*., 1997). These properties seem to be dependent on the ability of the N-terminal domain of H-NS to form oligomers (Ueguchi *et al*., 1996; Williams *et al*., 1996; Spurio *et al*., 1997). This region is predicted to be highly α-helical (Dorman *et al*., 1999; Bertin *et al*., 1999; Ceschini *et al*., 2000; Smyth *et al*., 2000), whereas the C-terminal domain, which has been resolved by nuclear magnetic resonance (NMR; Shindo *et al*., 1995), is a mixed $\alpha-\beta$ structure.

In *E. coli*, *hns* expression is growth phase-dependent and is subjected to autorepression (Atlung and Ingmer, 1997). Cold shock increases H-NS synthesis in *E. coli* (La Teana *et al*., 1991), and the synthesis of the orthologous protein, VicH, in *Vibrio cholerae* (Tendeng *et al*., 2000). Mutations in *hns* result in various phenotypes, because its product is involved in the regulation of apparently unlinked

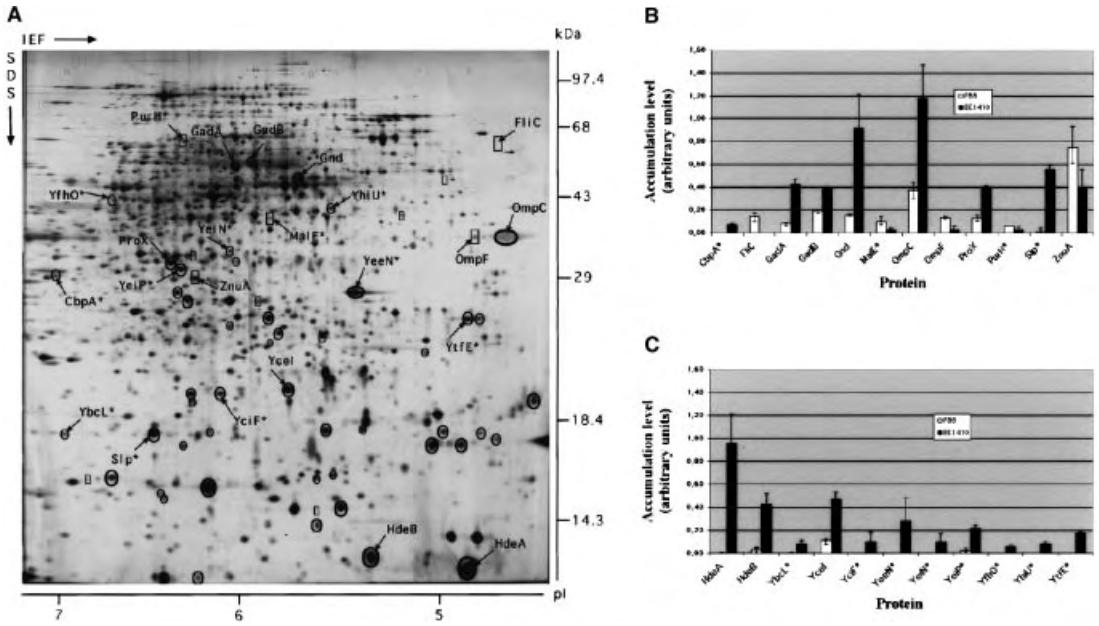

Fig. 1. Two-dimensional silver-stained map of the H-NS-deficient strain, BE1410, and comparative analysis of the level of accumulated proteins in the wild-type and *hns* mutant strains. Two-dimensional gel electrophoresis (A) was carried out as described in *Experimental procedures*. The 49 proteins represented by a circle and the 11 proteins represented by a rectangle correspond to polypeptides whose accumulation level was increased and decreased, respectively, in all experiments by at least a factor of two in BE1410 compared with the FB8 wild-type strain. The 23 identified spots are named and indicated by an arrow; those characterized by mass spectrometry are indicated by *. Protein levels are expressed as percentage volume, which corresponds to the percentage ratio between the volume of a single spot and the total volume of all spots present in a gel. The accumulation level of proteins with known function (B) and of putative or unknown function (C) in the wild-type strain (□) and in the *hns* mutant (■) was quantified using the MELANIE II software (Bio-Rad). The data are the mean values ± standard deviations of four independent experiments. The BLASTP program and the Prosite patterns database were used to compare the amino acid sequences of proteins with putative or unknown function (C) with the updated non-redundant databases. HdeA (P26604) has been proposed to have a chaperone-like function in extremely acidic conditions in pathogenic enteric bacteria. YeiN, YeeN and YtfE were similar to proteins of unknown function from *Thermotoga maritima*, *B. subtilis* and *Haemophilus influenzae*, respectively. YbcL belongs to the UPF0098 protein family, which comprises proteins of unknown function from *Mycobacterium tuberculosis* and *Archeoglobus fulgidus*. The YeiP (P33028) sequence was similar to elongation factors from various bacteria and contained the elongation factor P signature (PS01275); YfhO (P39171) was similar to pyridoxal phosphate enzymes and contained the corresponding signature (class V pyridoxal phosphate attachment site, PS00595). YhiU (P37636) was similar to the acriflavin resistance protein from *E. coli* and contained the prokaryotic membrane lipoprotein lipid attachment site (PS00013).

genes (Atlung and Ingmer, 1997; Bertin *et al.*, 1999). The control of the *proU* (Jordi and Higgins, 2000), *rrnB* (Schröder and Wagner, 2000), *bglGFB* (Caramel and Schnetz, 1998) and *flhDC* (Soutourina *et al.*, 1999) operons by H-NS has been studied extensively. Despite this, the function of H-NS in bacterial metabolism remains unclear. To elucidate the physiological role of this nucleoid-associated protein, we compared both the proteome and the transcriptome of an H-NS-deficient strain and its wild-type parent. These large-scale methods provided a wider view of the global regulation of gene expression in bacteria in response to multiple stresses.

Results

Comparative analysis of two-dimensional protein patterns of wild-type and H-NS-deficient strains

A representative pattern of silver nitrate-stained proteins extracted from *hns* mutant strain BE1410 and the quantitative differences in levels of accumulated proteins between BE1410 and the wild-type strain, FB8, are shown in Fig. 1. The cellular content of at least 60 proteins was altered in the H-NS-deficient strain: the amount of 49 polypeptides was increased and that of 11 polypeptides was decreased in the *hns* mutant strain compared with the wild type (Fig. 1A). These observations substantiate and extend our recent data (Laurent-Winter *et al.*, 1997; Bertin *et al.*, 1999; Soutourina *et al.*, 1999). To ensure that these modifications resulted from the *hns* mutation itself and not from any alteration depending on the genetic context, identical experiments were carried out with the *E. coli* K-12 reference strain MG1655 and its *hns* derivative. The differences in protein profiles between the wild-type and *hns* mutant strains were similar to those observed in FB8 and BE1410 (data not shown). Twenty-three spots, with molecular masses ranging from 18 to 100 kDa and pI of 4–7, accumulated to different levels in the two strains and were characterized by comparison with the *E. coli* reference

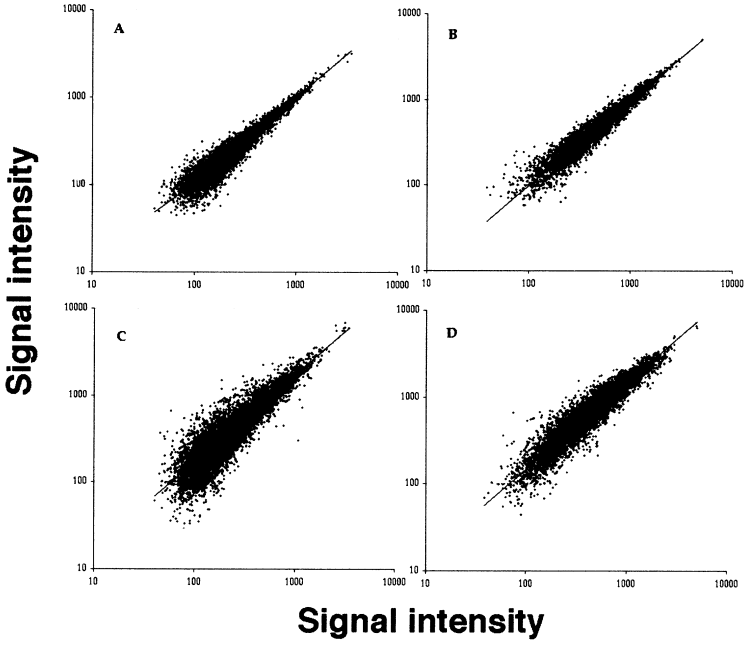

Fig. 2. Comparison of signal intensity measured on DNA arrays from dot duplicates and from independent hybridizations. The reproducibility of the results obtained with 1 μg (A and C) and 10 μg of total RNA (B and D) extracted from the *hns* mutant was assessed before subtraction of the background.
A and B. Comparison of signal intensity for pairs of dots corresponding to each gene.
C and D. Comparison of signal intensity for each gene in two independent hybridizations. Similar results were obtained from wild-type strain cDNA hybridization (data not shown).

map (ftp://ncbi.nlm.nih.gov/repository/ECO2DBASE) or by mass spectrometry (Fig. 1B and C).

The cellular content of several proteins whose accumulation is known to be controlled by H-NS was altered in the *hns* mutant strain BE1410 (Fig. 1A). These included ProX, Gnd, GadA and HdeA. Mass spectrometry identified several additional proteins. For example, CbpA, a curved DNA-binding protein induced by starvation (Richmond *et al*., 1999), showed an increased accumulation in the *hns* mutant (Fig. 1B). Similarly, eight hypothetical proteins of unknown function were accumulated to increased levels in the *hns* mutant (Fig. 1C). The BLASTP program (Altschul *et al*., 1990) and the Prosite pattern database (Hofmann *et al*., 1999) were used to compare the amino acid sequences of these proteins with those in the updated non-redundant databases: YeiP, YfhO and YhiU were similar to proteins of known function (Fig. 1C).

Expression profiling of wild-type and H-NS-deficient strains

H-NS has been described as a transcriptional regulator (Atlung and Ingmer, 1997). Therefore, to study the effect of this protein on the whole *E. coli* genome, RNA was isolated from BE1410 and its wild-type counterpart and analysed using DNA arrays spotted with the whole gene set of *E. coli*. To account for unspecific variations, experiments were carried out using at least three independent RNA preparations, from which at least two hybridizations were performed and two different sets of DNA arrays. Furthermore, to study the effect of H-NS on genes expressed at a low level, a second set of hybridizations was carried out with 10 times more (10 μg) total RNA. Comparison of the signal intensity of arrays from duplicates or from independent hybridizations (see *Experimental procedures*) showed that the results were highly reproducible (Fig. 2). A representative overlay of *hns* mutant and wild-type patterns is shown in Fig. 3. The numerous differences between wild-type and *hns* mutant strains show that H-NS has a major effect on gene expression. In particular, strongly depressed genes identified by this method (Fig. 3) encoded proteins that were shown to be accumulated to high levels on two-dimensional gels (Fig. 1). Expression intensities were above the background level for 2986 genes, which is comparable with previous results obtained with specific oligonucleotide primers (Tao *et al*., 1999) or with random hexamers (Arfin *et al*., 2000). A non-parametric statistical test (see *Experimental procedures*) showed that the expression level of about 250 genes was significantly different in the *hns* mutant strain compared with the wild type (P-value ≤ 0.05). Of these H-NS-regulated genes, approximately 20% had a well-known or putative function in processes such as transcription and translation. Over 35% were regulated in response to multiple environmental conditions or were involved in cell envelope composition (Fig. 4). The genes whose expression level differed by a factor ≥ 2 between the wild-type and mutant strains are listed in Table 1.

Regulation of genes of unknown function by H-NS

Most genes encoding hypothetical proteins (Table 1)

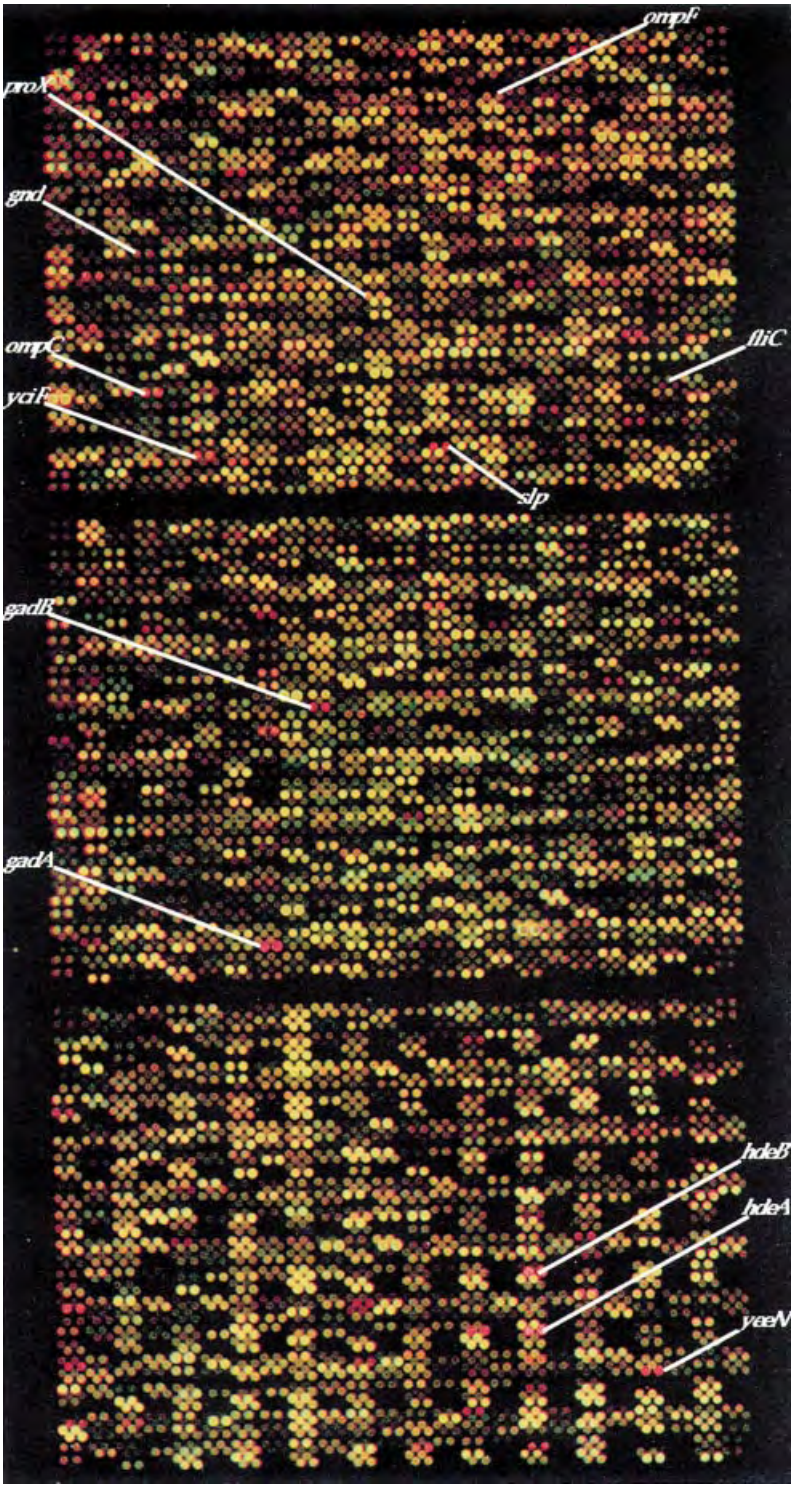

Fig. 3. Overlay of DNA arrays hybridized with wild-type or *hns* mutant cDNA. Variations in gene expression were measured with DNA arrays containing the 4290 open reading frames (ORFs) of the *E. coli* genome hybridized with cDNA probes generated from RNA extracted from wild-type and *hns* BE1410 mutant strains. Macroarrays were scanned as described in *Experimental procedures*, and the ADOBE PHOTOSHOP F1-4.0 software was used to produce the overlay of representative macroarrays. The macroarray hybridized with the *hns* mutant cDNA probes was coloured red and that with the wild-type cDNA probes was coloured green. Genes whose expression was not modified in H-NS-deficient and wild-type strains are coloured yellow, and those induced or repressed in the *hns* mutant are coloured red or green respectively. Genes whose product showed an altered accumulation level between wild-type and *hns* strains (Fig. 1) are indicated by an arrow and named.

were tentatively characterized using the BLASTP program to compare the amino acid sequence of their product with the updated non-redundant databases (Altschul *et al.*, 1990). The pattern database Prosite from the ExPASy server (Hofmann *et al.*, 1999) was used to search for conserved patterns. Twenty-two gene products were similar to proteins of well-known function from a variety of organisms (Table 1). Sixteen gene products were not similar to any of the proteins present in the databases, and 18 were predicted to be similar to proteins of unknown function from different microorganisms.

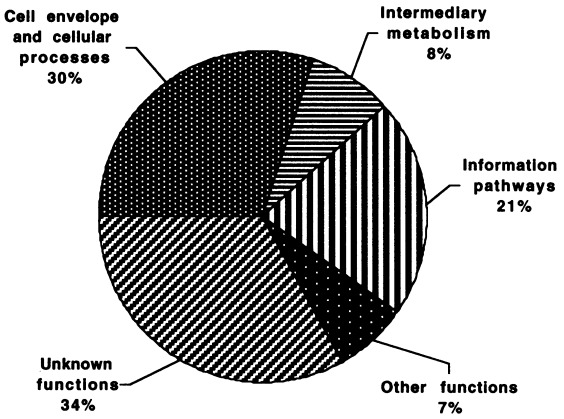

Fig. 4. Functional classification of H-NS-regulated *genes* identified by expression profiling on DNA arrays. Genes that showed a differential expression between wild-type and mutant strain (*P*-value ≤ 0.05) were classified according to their function (Moszer, 1998).

Cell envelope components controlled by H-NS

The cellular localization of H-NS-regulated gene products was determined using the Niceprot database (http://www.expasy.ch/cgi-bin/niceprot.pl) or predicted using the PSORT program (http://psort.nibb.ac.jp:8800/). Approximately half the proteins encoded by genes controlled by H-NS were located in or associated with membranes or were present in the periplasmic space (Table 1). For example, there were higher mRNA levels of 10 genes belonging to an operon involved in the biosynthesis of capsular polysaccharide colanic acids, such as *gmd* and *wzc* (Whitfield and Roberts, 1999), and 10 genes involved in lipopolysaccharide (LPS) biosynthesis, such as *rfaI* and *wbbJ*, in an H-NS-deficient background. The synthesis of *gspG* transcripts, which encodes a general secretion pathway protein, increased over 10-fold, whereas the greatest decrease in mRNA levels in the mutant strain corresponded to seven of the flagellar cascade genes. These encode flagellum structural components, such as flagellin (encoded by *fliC*). This observation is consistent with the proteome analysis (Fig. 1) and also supports a key role for H-NS in the positive regulation of motility and flagellum synthesis *in vivo* (Bertin *et al*., 1994; Soutourina *et al*., 1999). Similarly, several genes encoding proteins involved in fimbriae (*fimB* and *fimI*) and curli biosynthesis (*csgA*) were induced in the *hns* mutant (Table 1). Finally, five genes encoding hypothetical proteins similar to fimbriae and *ompX*, which encodes a porin-like protein involved in virulence in different microorganisms, were upregulated in the *hns* mutant (Table 1).

Effect of H-NS on DNA-binding protein-encoding genes

Several genes that are upregulated in the *hns* mutant code for proteins with nucleic acid-binding properties (Table 1). These include *dps* whose product appears to protect DNA from oxidative damage in the stationary phase (Bridges and Timms, 1998), *stpA*, which encodes an H-NS paralogue in *E. coli* (Zhang *et al*., 1996), and the DNA-binding protein, Lrp, structural gene (Newman *et al*., 1992). We observed more than a threefold increase in the stationary phase sigma factor mRNA, suggesting that, in addition to its role in stabilizing RpoS (Barth *et al*., 1995; Yamashino *et al*., 1995), H-NS could also directly or indirectly regulate the expression of the *rpoS* gene (Table 1). The mRNA levels of three regulators from two-component systems were also increased in the *hns* mutant: *phoP* from the PhoPQ system, which is involved in the response to magnesium limitation (Kato *et al*., 1999); *evgA* from the EvgAS system, which is similar to the *Bordetella pertussis bvgA* gene; and *yedW*, the regulator from the two-component system YedVW (Table 1). Finally, five gene products of unknown function (*ycgE*, *ydeO*, *yhiW*, *yhiX and yjhI*) were predicted to encode regulatory proteins belonging to the AraC, IclR and MerR families (Table 1).

Changes in the levels of mRNAs of starvation-induced proteins in the hns *mutant strain*

Some genes induced by oxygen starvation, such as *cydAB*, which encodes the cytochrome *d* ubiquinol oxidase complex (Cotter *et al*., 1990), and *appB*, which encodes a cytochrome *bd* II oxidase (Sturr *et al*., 1996), showed increased expression in the *hns* mutant background (Table 1). The transcript levels of at least three genes involved in the carbon starvation response were also increased in the *hns* mutant; these included *gnd*, whose product is involved in the hexose monophosphate shunt by converting 6-phospho-D-gluconate into D-ribulose 5-phosphate (Sprenger, 1995), and *slp*, which encodes a lipoprotein that stabilizes the outer membrane (Alexander and St John, 1994). This was consistent with our two-dimensional data (Fig. 1). Finally, the mRNA levels of 21 genes encoding ribosomal proteins, which are repressed by growth in minimal medium (Gausing, 1977; Tao *et al*., 1999), were decreased in the mutant strain (Table 1).

Increased resistance to high osmolarity and low pH in an H-NS-deficient strain

DNA array experiments suggested that many H-NS-regulated genes encode proteins that are involved in responses to environmental stresses. Several genes are involved in the response to high osmolarity (Csonka and Epstein, 1996); for example, *ompC and ompF*, which both encode porins, and *proX*, which encodes a glycine-betaine binding protein, consistent with our two-dimensional data (Fig. 1) and previous studies (Atlung and Ingmer, 1997). The *osmC* gene, which is induced by high

Table 1. Genes differentially expressed between *E. coli* K-12 strains FB8 (wild type) and BE1410 (*hns* mutant).

Gene[ab]	Expression ratio BE1410/FB8[cd]	P-value	SWISSPROT and TrEMBL accession no.[e]	Function	Subcellular localization[f]
A	Cell envelope and cellular processes				
appB[b]	2.67	2E-2	P26458	Cytochrome *bd* I oxidase subunit II	Integral inner membrane
caiT	− 2.27	9E-3	P31553	Probable PMF-driven uptake of carnitine and betaines	Integral inner membrane
cirA[b]	−2.33	4E-2	P17315	Colicin I receptor	Outer membrane
cpsB	2.70	5E-2	P24174	Mannose-1-phosphate guanylyltransferase	Cytoplasmic[f]
csgA[ab]	2.83	4E-2	P28307	Major curlin subunit	Outer membrane
cydA[b]	2.45	5E-2	P11026	Cytochrome *d* ubiquinol oxidase subunit I	Integral inner membrane
cydB	9.26	5E-2	P11027	Cytochrome *d* ubiquinol oxidase subunit II	Integral inner membrane
cyoA[b]	10.20	5E-2	P18400	Cytochrome *o* subunit II	Integral inner membrane
feoB	4.26	5E-2	P33650	Ferrous iron transport protein B	Integral inner membrane
fimB[ab]	12.39	5E-3	P04742	Fimbriae recombinase	Cytoplasmic probable
fimI	6.61	2E-2	P39264	Fimbrin-like protein	Outer membrane
flgA	− 2.44	4E-2	P75933	Basal body P-ring formation protein	Periplasmic probable
flgE	− 2.17	2E-2	P75937	Flagellar hook subunit protein	Cytoplasmic[f]
flgH	− 2.86	5E-2	P75940	Basal body L-ring protein	Outer membrane
flgI	− 2.00	3E-2	P75941	Basal body P-ring protein	Periplasmic
flhA	− 2.00	9E-3	P76298	Flagellar synthesis	Integral inner membrane
fliC[ab]	−12.50	9E-4	P04949	Flagellin	Extracellular
fliR	− 1.97	1E-3	P33135	Flagellar synthesis	Integral inner membrane
gadC[b]	13.67	5E-3	P39183	Putative amino acid antiporter	Integral inner membrane probable
glf	2.53	1E-2	P37747	UDP-galactopyranose mutase	Inner membrane[f]
gmd	8.69	3E-2	P32054	GDP-mannose 4,6-dehydratase	Inner membrane[f]
gmm	5.60	5E-2	P32056	Hydrolase	Cytoplasmic[f]
gspE	3.83	5E-2	P45759	Probable general secretion pathway protein E	Cytoplasmic probable
gspG	16.97[c]	5E-2	P41442	Probable general secretion pathway protein G	Periplasmic[f]
gspM	3.05	5E-2	P36678	Probable general secretion pathway protein M	Inner membrane probable
gspO	4.20	5E-2	P25960	Type 4 prepilin-like protein leader peptide processing enzyme	Integral inner membrane probable
ompC[a]	3.24	1E-3	P06996	Porin	Integral outer membrane
ompF[ab]	−3.85	3E-3	P02931	Porin	Integral outer membrane
ompN	2.50	2E-2	P77747	Similar to Q56111, outer membrane protein S2 (*Salmonella typhimurium*); belongs to the OmpC/PhoE family of porins	Integral outer membrane
ompX	3.58	5E-3	P36546	Similar to P16454, attachment invasion locus protein precursor (*Yersinia enterocolitica*); belongs to the Ail/OmpX/PagC/Lom family PS00694 and PS000695: Enterobacterial virulence outer membrane protein signatures	Integral outer membrane
osmC[ab]	5.89	2E-3	P23929	Similar to osmotically inducible protein C (*Deinococcus radiodurans*)	Inner membrane[f]
rfaI	5.25[c]	3E-2	P27128	Lipopolysaccharide 1,3-galactosyltransferase	Periplasmic[f]
rfaJ	3.97[c]	2E-2	P27129	Lipopolysaccharide 1,2-glucosyltransferase	Inner membrane[f]
rfaK	4.52[c]	5E-2	P27242	Lipopolysaccharide 1,2-*N*-acetylglucosaminetransferase	Peripheral membrane
rfaL	2.00	2E-2	P27243	Lipopolysaccharide core biosynthesis, O-antigen ligase	Integral membrane
rfaS	3.50[c]	4E-2	P27126	Lipopolysaccharide core biosynthesis protein	Inner membrane[f]
rfaY	2.00	5E-2	P27240	Lipopolysaccharide core biosynthesis	Cytoplasmic[f]
slp[b]	22.96	4E-4	P37194	Lipoprotein	Attached to the outer membrane by a lipid anchor
tatA	2.00	2E-2	P27856	Translocation of periplasmic proteins, preservation of structures and ligands	Integral membrane
tatE	2.30	5E-2	P25895	Translocation of periplasmic proteins, preservation of structures and ligands	Inner membrane[f]
wbbI	2.75	1E-2	P37749	Involved in lipopolysaccharide biosynthesis	Cytoplasmic probable
wbbJ[b]	4.20[c]	5E-2	P37750	Putative lipopolysaccharide O-acetyltransferase	Inner membrane[f]

Table 1. *continued*

Gene[ab]	Expression ratio BE1410/FB8[cd]	P-value	SWISSPROT and TrEMBL accession no.[e]	Function	Subcellular localization[f]
wbbK	2.15	2E-2	P37751	Lipopolysaccharide glycosyltransferase, transfer of UDP-galF and UDP-glucose	Inner membrane-associated probable
wcaA	2.00	5E-2	P77414	Involved in lipopolysaccharide biosynthesis	Cytoplasmic[f]
wcaC	6.64	5E-2	P71237	Putative glycosyl transferase	Inner membrane[f]
wcaE	8.70	5E-2	P71239	Putative glycosyl transferase	Cytoplasmic[f]
wcaF	4.69	5E-2	P71240	Putative acetyltransferase	Cytoplasmic[f]
wcaI	6.30	5E-2	P32057	Putative glycosyl transferase	Inner membrane[f]
wcaJ	3.94	5E-2	P71241	UDP-glucose lipid carrier transferase	Inner membrane[f]
wzb	6.62	5E-2	P77153	Probable low-molecular-weight protein tyrosine phosphatase	Inner membrane[f]
wzc[b]	7.12	5E-2	P76387	Putative membrane-associated ATP hydrolase	Inner membrane[f]
yadN	23.12[cd]	5E-2	P37050	Similar to P12267, type 3 fimbrial protein MrkA precursor (*Klebsiella pneumoniae*)	Outer membrane[f]
yagX	2.52	5E-2	P77802	Similar to P25733, CFA/I fimbrial subunit C precursor (*E. coli*)	Outer membrane[f]
yaiP	22.70[cd]	5E-2	P77760	Similar to Q54066, IcaA (*Staphylococcus epidermidis*)	Inner membrane[f]
ycbQ	8.86	1E-3	P75855	Similar to P12903, fimbrial subunit type 1 precursor (*K. pneumoniae*)	Outer membrane[f]
ycgH	2.50	2E-2	AE000215[e]	Similar to AE000248 putative ATP-binding component of a transport system and adhesin protein (*E. coli*)	Outer membrane[f]
yddA	10.06	5E-3	P31826	Similar to Q57335, Y036 hypothetical ABC transporter ATP-binding protein (*Haemophilus influenzae*) PS00211 and PS00017: ATP-GTP binding site motif A and ABC transporter family	Integral membrane probable
ydeT	43.47[c]	1E-2	AE000247[e]	Similar to P30130 outer membrane protein; export and assembly of type 1 fimbriae (*E. coli*)	Cytoplasmic[f]
yedV	6.17	1E-3	P76339	Similar to O44007, sensor protein CzcS (*Ralstonia eutropha*)	Integral inner membrane probable
yhhZ[b]	2.56	5E-3	P46855	Similar to P80693 MajE, the major exported protein (*Pseudomonas syringae*)	Extracellular probable
yjdE	6.13	3E-2	P39269	Similar to P23891, probable cadaverine/lysine antiporter (*E. coli*)	Integral inner membrane probable
B	*Intermediary metabolism*				
gadA[ab]	56.00	4E-3	P80063	Glutamate decarboxylase-α	Cytoplasmic probable
gadB[ab]	56.32	4E-3	P28302	Glutamate decarboxylase-β	Cytoplasmic probable
gltD	2.76	5E-2	P09832	Glutamate synthase β-subunit	Cytoplasmic
gnd[a]	3.57	5E-3	P00350	6-Phosphogluconate dehydrogenase	Inner membrane[f]
pflB	2.83	5E-2	P09373	Pyruvate formate lyase 1	Cytoplasmic[f]
tdcE	2.75	4E-2	P42632	Pyruvate formate lyase or ketobutyrate formate lyase	Cytoplasmic by similarity
ugd[b]	3.24	2E-2	P76373	UDP-glucose-6-dehydrogenase	Inner membrane[f]
ydeP[b]	7.47	6E-3	P77561	Similar to P06131, formate dehydrogenase α-chain (*Methanobacterium formicicum*)	Cytoplasmic[f]
ydiO	32.70[c]	5E-2	P76200	Similar to P31571, probable carnitine operon oxidoreductase (*E. coli*) PS00072 and PS00073: Acyl-coA dehydrogenases signatures 1 and 2	Cytoplasmic[f]
ydjH[b]	4.60	4E-2	P77493	Similar to P44331, ribokinase RbsK homologue (*H. influenzae*) PS00583 and PS00584: carbohydrate kinase signatures 1 and 2	Inner membrane[f]
yfdW	8.08	1E-2	P77407	Similar to U82167 formyl-CoA transferase (*Oxalobacter formigenes*)	Cytoplasmic[f]
C	*Information pathways*				
appY[a]	2.74	4E-3	P05052	Regulatory protein	Cytoplasmic
aslB[b]	5.70	2E-2	P25550	Putative arylsulphatase regulatory protein	Cytoplasmic[f]
evgA	7.35[d]	2E-2	P30854	Regulator of the two-component regulatory system EvgA/EvgS	Cytoplasmic probable
gltF[b]	8.49	4E-3	P28721	Putative regulatory protein	Cytoplasmic
hha	20.75	7E-3	P23870	Haemolysin expression modulating protein	Cytoplasmic[f]
leuO[a]	8.69	2E-2	P10151	LysR regulatory protein	Cytoplasmic
ligA	7.62	5E-2	P15042	DNA ligase	Cytoplasmic[f]

Table 1. *continued*

Gene[ab]	Expression ratio BE1410/FB8[cd]	P-value	SWISSPROT and TrEMBL accession no.[e]	Function	Subcellular localization[f]
lrp[a]	30.69[c]	5E-2	P19494	Leucine-responsive regulatory protein	Cytoplasmic
phoP[b]	2.72	1E-2	P23836	Regulator of the two-component system PhoP/PhoQ	Cytoplasmic
pinQ[b]	2.69	7E-3	P77170	DNA invertase	Cytoplasmic[f]
pinR[b]	8.72	3E-3	P77574	DNA invertase	Cytoplasmic[f]
rcsA[ab]	2.60	1E-3	P24210	Regulatory protein	Cytoplasmic
rplA[b]	−3.70	2E-2	P02384	50S ribosomal subunit protein L1	Cytoplasmic
rplB	− 2.22	3E-2	P02387	50S ribosomal subunit protein L2	Cytoplasmic
rplC	− 2.00	1E-2	P02386	50S ribosomal subunit protein L3	Cytoplasmic
rplF	− 2.00	1E-2	P02390	50S ribosomal subunit protein L6	Cytoplasmic
rplI[b]	−3.33	5E-2	P02418	50S ribosomal subunit protein L9	Cytoplasmic
rplK	− 2.22	4E-3	P02409	50S ribosomal subunit protein L11	Cytoplasmic
rplL	− 2.08	2E-3	P02392	50S ribosomal subunit protein L7/L12	Cytoplasmic
rplN	− 4.00	5E-2	P02411	50S ribosomal subunit protein L14	Cytoplasmic
rplO	− 2.00	4E-4	P02413	50S ribosomal subunit protein L15	Cytoplasmic
rplQ	− 2.70	2E-2	P02416	50S ribosomal subunit protein L17	Cytoplasmic
rplW	− 2.77	2E-2	P02424	50S ribosomal subunit protein L23	Cytoplasmic
rplX	− 3.13	1E-2	P02425	50S ribosomal subunit protein L24	Cytoplasmic
rpmC	− 2.08	8E-3	P02429	50S ribosomal subunit protein L29	Cytoplasmic
rpmF	− 2.00	6E-3	P02435	50S ribosomal subunit protein L32	Cytoplasmic
rpmG	− 2.50	5E-2	P02436	50S ribosomal subunit protein L33	Cytoplasmic
rpoS[a]	3.75	5E-2	P13445	RNA polymerase sigma factor 38	Cytoplasmic
rpsH	− 2.04	5E-2	P02361	30S ribosomal subunit protein S8	Cytoplasmic
rpsI	− 2.99	2E-2	P02363	30S ribosomal subunit protein S9	Cytoplasmic
rpsM	− 2.00	2E-2	P02369	30S ribosomal subunit protein S13	Cytoplasmic
rpsN	− 2.17	5E-2	P02370	30S ribosomal subunit protein S14	Cytoplasmic
rpsQ	− 2.50	4E-2	P02373	30S ribosomal subunit protein S17	Cytoplasmic
rpsS[b]	−2.86	2E-2	P02375	30S ribosomal subunit protein S19	Cytoplasmic
stpA[a]	9.76	5E-2	P30017	DNA-binding protein	Cytoplasmic
ycgE	14.00	4E-2	P75989	Similar to P33358, YehV putative transcriptional regulator (*E. coli*) PS00552: Bacterial regulatory protein MerR family signature	Cytoplasmic[f]
ydeO[b]	3.87	2E-2	P76135	Similar to P05052, AppY regulatory protein (*E. coli*) PS00041: Bacterial regulatory proteins AraC family signature	Cytoplasmic
yedW[b]	35.36[c]	5E-2	P76340	Similar to O44006, regulatory protein CzcR (*Ralstonia eutropha*)	Cytoplasmic probable
yfiA	3.72	4E-2	P11285	Similar to U32711, sigma54 modulation protein, putative (*H. influenzae*)	Cytoplasmic[f]
yhiW[b]	32.41[c]	2E-2	P37638	Similar to P05052, AppY regulatory protein (*E. coli*) PS00041: Bacterial regulatory proteins AraC family signature	Cytoplasmic[f]
yhiX[b]	13.92	4E-4	P37639	Similar to P10805, regulatory protein EnvY (*E. coli*) PS00041: Bacterial regulatory proteins AraC family signature	Cytoplasmic[f]
yjhI[b]	24.07[c]	4E-2	P39360	Similar to P76268, KdgR transcriptional regulator (*E. coli*) PS01051: Bacterial regulatory proteins, IclR family signature	Cytoplasmic[f]
D	*Other functions*				
appA[b]	2.50[d]	3E-2	P07102	Acid phosphatase, pH 2.5	Periplasmic
cspB	4.10	5E-2	P36995	Similar to cold shock protein PS00352: Cold shock DNA-binding domain signature	Cytoplasmic probable
cspC	4.73	1E-2	P36996	Similar to cold shock protein PS00352: Cold shock DNA-binding domain signature	Cytoplasmic probable

Table 1. *continued*

Gene[ab]	Expression ratio BE1410/FB8[cd]	P-value	SWISSPROT and TrEMBL accession no.[e]	Function	Subcellular localization[f]
cspE	− 3.03	1E-3	P36997	Similar to cold shock protein PS00352: Cold shock DNA-binding domain signature	Cytoplasmic probable
cspG[b]	9.10	5E-2	Q47130	Similar to cold shock protein PS00352: Cold shock DNA-binding domain signature	Cytoplasmic probable
cspI[b]	8.71	8E-3	P77605	Similar to cold shock protein PS00352: Cold shock DNA-binding domain signature	Cytoplasmic probable
dps	30.71[c]	3E-2	P27430	DNA protection during starvation protein	Cytoplasmic probable
ftn	3.54	8E-3	P23887	Iron storage protein	Cytoplasmic
groL[a]	2.40	3E-2	P06139	Chaperone for assembly of enzyme complexes	Cytoplasmic
hdeA[ab]	48.40	4E-4	P26604	Putative chaperone-like protein involved in low pH resistance	Periplasmic[f]
hdeB[ab]	154.83	4E-4	P26605	Similar to *hdeB* (*Shigella flexneri*), involved in low pH resistance	Periplasmic[f]
proX[ab]	3.50	7E-3	P14177	Glycine-betaine binding protein	Periplasmic
spy	8.83	5E-2	P77754	Spheroplast protein Y	Periplasmic
yddT	2.92	4E-3	P77790	Similar to vancomycin resistance protein VanX (*E. coli*)	Cytoplasmic[f]
E	*Unknown proteins*				
hdeD[ab]	4.80	4E-3	P26603	Unknown function	Periplasmic[f]
yagK[b]	24.78[c]	4E-2	P77657	Similar to P52125, YfjJ hypothetical protein (*E. coli*)	Cytoplasmic[f]
yagY	8.34	5E-2	P77188	Similar to P77263, YagV hypothetical protein (*E. coli*)	Periplasmic[f]
yaiS[b]	25.37[c]	3E-2	P71311	Similar to P42981, YpjG hypothetical protein (*B. subtilis*)	Cytoplasmic[f]
ybaJ	4.14	3E-2	P37611	Unknown function	Cytoplasmic[f]
ybcL	3.41	5E-2	P77368	Similar to AE004189, hypothetical conserved protein (*V. cholerae*)	Outer membrane[f]
ybgE[b]	36.64[c]	4E-2	P37343	PS00013: Membrane lipoprotein lipid attachment site signature	Inner membrane[f]
ycbW	3.00	2E-2	P75862	Unknown function	Inner membrane[f]
yccA	5.71	3E-2	P06967	Similar to O25578, hypothetical membrane protein (*Helicobacter pylori*) PS01243: uncharacterized protein family UPF005 signature	Integral membrane probable
ycdT[b]	2.97	1E-3	P75908	Similar to P76236, YeaI hypothetical protein (*E. coli*)	Integral membrane probable
ycgX[b]	3.02[c]	3E-2	P75988	Similar to P76156, YdfO hypothetical protein (*E. coli*)	Cytoplasmic[f]
yciF[b]	2.31	4E-4	P21362	Unknown function	Cytoplasmic[f]
yciG[b]	6.26	3E-3	P21361	Similar to P56614, YmdF hypothetical protein (*E. coli*) PS00017: ATP-GTP binding site motif A	Cytoplasmic[f]
ydbA	17.62	8E-3	P33666	Unknown function	Cytoplasmic[f]
ydbD[b]	2.93	5E-2	P25907	Unknown function	Periplasmic[f]
ydcD[b]	5.03	1E-3	P31991	Unknown function	Inner membrane[f]
ydgK	4.02	5E-2	P76180	Unknown function	Inner membrane probable
yeeN[b]	6.31	4E-4	P76351	Belongs to the UPF0082 family	Cytoplasmic[f]
yegR[b]	13.33	5E-3	P76406	Unknown function	Inner membrane[f]
yegZ	4.08	2E-2	AE000298[e]	Similar to P10312 D protein GPD bacteriophage P2	Cytoplasmic[f]
yehA[b]	6.32	5E-2	P33340	Unknown function	Cytoplasmic probable
ygaP[b]	6.22	5E-2	P55734	Similar to P73801 hypothetical protein (*Synechocystis* sp.)	Inner membrane[f]
ygbF	22.95[c]	2E-2	P45956	Unknown function	Cytoplasmic[f]
ygbT	2.29	3E-2	Q46896	Unknown function	Cytoplasmic[f]
ygcF[b]	4.91	4E-2	P55139	Similar to D90908 hypothetical protein (*Synechocystis* sp.)	Cytoplasmic[f]
ygcJ	4.04	5E-2	Q46899	Unknown function	Cytoplasmic[f]
ygcK	4.35	2E-2	P76632	Unknown function	Cytoplasmic[f]
ygcL[b]	8.17	2E-2	Q46901	Unknown function	Inner or outer membrane[f]
yhiE[b]	11.08	4E-4	P29688	Similar to P37195 YhiF hypothetical protein (*E. coli*)	Cytoplasmic[f]

Table 1. *continued*

Gene[ab]	Expression ratio BE1410/FB8[cd]	P-value	SWISSPROT and TrEMBL accession no.[e]	Function	Subcellular localization[f]
yhiF	12.46	3E-2	P37195	Similar to P29688 YhiE hypothetical protein (*E. coli*)	Inner membrane[f]
yhiM[b]	17.51	4E-4	P37630	Unknown function	Inner membrane[f]
ykgB	40.86[c]	4E-2	P75685	Similar to gbAAB18029, hypothetical protein (*H. influenzae*)	Integral inner membrane probable
ynaE	6.14	4E-4	P76073	Similar to P76154 YdfK hypothetical protein (*E. coli*)	Cytoplasmic[f]
yncI[b]	15.30	9E-4	AE000243[e]	Similar to P28912 H repeat-associated protein (*E. coli*)	Inner membrane[f]

The results obtained are representative of at least four hybridizations from at least three independent RNA extractions. Only genes with a *P*-value ≤ 5E-2 in a Wilcoxon statistical test and showing a factor ≥ 2 differential expression between wild-type and *hns* strains were classified according to their function, i.e. cell envelope and cellular processes (A), intermediary metabolism (B), information pathways (C), other functions (D) or unknown functions (E). Genes that were previously shown to be regulated by H-NS (Atlung and Ingmer, 1997; Laurent-Winter *et al.*, 1997) are indicated by **a** in the first column. The expression level of the genes indicated by **b** was also analysed with random hexamer primer-generated cDNAs synthesized from RNAs extracted by a rapid procedure and then hybridized on high-density filters designed in our laboratory. The differential expression measured between wild-type and mutant strains was similar to that obtained with Panorama arrays (F. Hommais, unpublished). An expression ratio measured from 10 μg of RNA is indicated by **c** in the second column, and genes whose expression is less than the background in one of the two strains are indicated by **d** in the third column. Columns four and five indicate protein accession number and protein function according to the SWISSPROT and TrEMBL databases (http://www.expasy.ch/SWISSPROT) or GenBank (**e**) respectively. The results from similarity searches carried out using the BLASTP program (http://www.expasy.ch/cgi-bin/BLASTNCBI pL?) against non-redundant updated databases are shown in column five. Protein subcellular localization (column six) came from the Niceprot database (http://www.expasy.ch/cgi-bin/niceprot.pl) or was predicted (**f**) using the PSORT software (http://psort.ims.u-tokyo.ac.jp/).

osmolarity, showed an increased mRNA level in the mutant strain. Finally, the *evgA* gene product (see above), which can induce *ompC* expression in an *envZ* mutant strain (Utsumi *et al.*, 1992), was upregulated in the mutant strain. Owing to the number of H-NS-regulated genes involved in osmoregulation (Table 1), we analysed the role of this regulatory protein in the response to osmotic stress. A fivefold increase in resistance to high ionic strength was measured in the *hns* mutant strain compared with the wild-type strain (Table 2A).

The *cadBA* operon and the *adiA* gene, which encode a lysine decarboxylase, an antiporter and an arginine decarboxylase, respectively, have been shown to be regulated by H-NS in rich medium under anaerobic conditions (Atlung and Ingmer, 1997). These genes are involved in the response to acid stress. Comparison of expression profiling (Table 1) showed that at least seven genes encoding proteins induced at low pH were increased in the *hns* mutant. For example, *gadC* (formerly called *xasA*) encodes a probable GABA/glutamate antiporter (De Biase *et al.*, 1999), and *gadA* and *gadB* encode two glutamate decarboxylases (Table 1). The *hdeA*-encoded protein has been proposed to have a chaperone-like function under extremely acidic conditions (Gajiwala and Burley, 2000). The corresponding increase in its mRNA level (Table 1) is consistent with the increased accumulation of its gene product observed on two-dimensional gels (Fig. 1). This prompted us to study the effect of the *hns* mutation on resistance to acidic stress via lysine, arginine or glutamate. The wild-type strain had a low level of resistance to acid stress in the presence of lysine or arginine, compared with that in the absence of any amino acid (Table 2A). This can be explained by the minimal growth medium and aerobic growth conditions used in this study (see above). In contrast, the presence of glutamate resulted in a moderate increase in acid resistance. Similarly, in the H-NS-deficient strain BE1410, a moderate increase in resistance was measured in the presence of lysine or arginine. Furthermore, a large increase in resistance (nearly 40% viable cells) was measured in the *hns* mutant strain in the presence of glutamate compared with that in the wild-type strain.

To investigate the regulation underlying this acid resistance in the *hns* mutant strain, we overexpressed *E. coli* genes from a genomic library in the wild-type strain FB8 subjected to acid stress, as described in *Experimental procedures*, and screened for an acid resistance phenotype in the presence of glutamate. Analysis of clones resistant to low pH allowed us to select pDIA567, which increased the resistance of the wild-type strain in the presence of glutamate to similar levels to that of an *hns* mutant (44.5% viable cells). Sequence determination of the DNA insert showed that this plasmid carries a

Table 2. High osmolarity and low pH resistance in wild-type and *hns* strains[a].

Growth conditions	Percentage survival[b]	
	FB8 (wild type)	BE1410 (*hns*)
A High osmolarity stress[c]		
3 M NaCl	2.6	12.5
B Acid stress[d]		
pH 2.5	≤ 0.01	0.9
pH 2.5 + 0.012% lysine	0.1	2.9
pH 2.5 + 0.012% arginine	0.2	13.0
pH 2.5 + 0.012% glutamate	4.1	38.0

a. Resistance measurement to both stresses was carried out as described in *Experimental procedures*. Viable bacterial cells were counted on plates.
b. Percentage survival is calculated as 100 × the number of cfu ml^{-1} remaining after acid or osmotic treatment divided by the initial cfu ml^{-1} at time zero. Values presented are representative of three independent experiments performed with the wild-type FB8 or the *hns* mutant BE1410 strains.
c. Cells were incubated in K5 medium supplemented with 0.4% glucose and 3 M NaCl.
d. Cells were incubated in M9 medium supplemented with 0.2% glucose and 0.012% amino acid.

fragment encompassing both *yhiX* and *gadA*. Castanie-Cornet *et al*. (1999) recently suggested that the over-expression of *gadA* could result in the acid resistance measured in the wild-type strain. The *yhiX* gene, whose expression was increased over 13-fold in the H-NS-defective strain (Table 1), encodes a protein similar to the AraC family of regulatory proteins. To determine whether this YhiX putative regulator could confer acid resistance, its structural gene was amplified by polymerase chain reaction (PCR) and then cloned and overexpressed from pDIA570 in FB8 wild-type strain. Remarkably, 55.3% of these bacteria survived exposure to acid stress in the presence of glutamate. This strongly suggests that this regulatory protein is involved in the control of bacterial adaptation to low pH in the presence of glutamate. Thus, the corresponding gene was renamed *gadX*. To test the hypothesis that *gadX* could play a role in the control of H-NS-regulated genes induced by low pH, we analysed the expression levels of some pH-regulated genes in a wild-type strain overexpressing *gadX*. DNA fragments corresponding to the whole protein coding sequence (CDS) of various pH-regulated genes were amplified by PCR and spotted onto nylon membranes in duplicate, and these membranes were hybridized with cDNA probes generated from 1 μg of RNA. Quantitative measurements showed that the level of *hdeD* transcripts increased by up to threefold (Fig. 5). More importantly, the expression of *gadA*/*gadB* and *gadC* genes, which are specifically involved in the response to low pH, increased over eightfold. This further supports a role for GadX in the positive regulation of genes involved in low pH response, especially in the presence of glutamate.

Discussion

Proteome and transcriptome analyses demonstrated that the nucleoid-associated protein, H-NS, plays a major role in bacterial physiology by directly or indirectly controlling the expression of many genes and/or the synthesis of their product (these data are accessable at the web site http://www.pasteur.fr/recherche/unites/RE6/H-NS/regulateur.HTM). In our growth conditions, we could visualize up to 1200 spots, of which the accumulation level of at least 5% was altered in the mutant strain (Fig. 1). Similarly, the expression level of nearly 5% of the *E. coli* 4290 CDSs was altered on DNA macroarrays (Fig. 3). The expression of over 80% of these genes was induced in the *hns* mutant strain, which further supports the role of H-NS as a repressor of gene expression. Of all the *E. coli* genome CDSs, many H-NS-regulated genes are involved in bacterial responses to multiple environmental conditions and/or in cell envelope composition. In addition, a number of genes that were up- or downregulated in the *hns* mutant have putative functions or have been described as hypothetical in databases (http://genolist.pasteur.fr/Colibri/). Our results are the first demonstration that these genes can be expressed, at least under some conditions. Similarity searches, pattern identification and cellular localization suggested that many of these genes encode proteins that are localized at the cell surface (Table 1). It

Fig. 5. Effect of *gadX* overexpression on pH-regulated genes. DNA fragments were amplified by PCR using primers specific for the 5′ and 3′ ends of the corresponding nucleotide sequence (Sigma–GenoSys Biotechnologies) and spotted in duplicate using a Q Pix arrayer (Genetix). Wild-type FB8 strains in the presence or not of pDIA570 overexpressing *gadX* were grown to $A_{600} = 0.5$ in minimal medium, pH 5.5, supplemented with 0.4% glucose and 0.012% glutamate. RNAs were extracted by a rapid procedure (I. Guillouard, unpublished), and cDNA probes were prepared with random hexamer primers (F. Hommais, unpublished). After hybridization, the results were quantified as described in *Experimental procedures*.

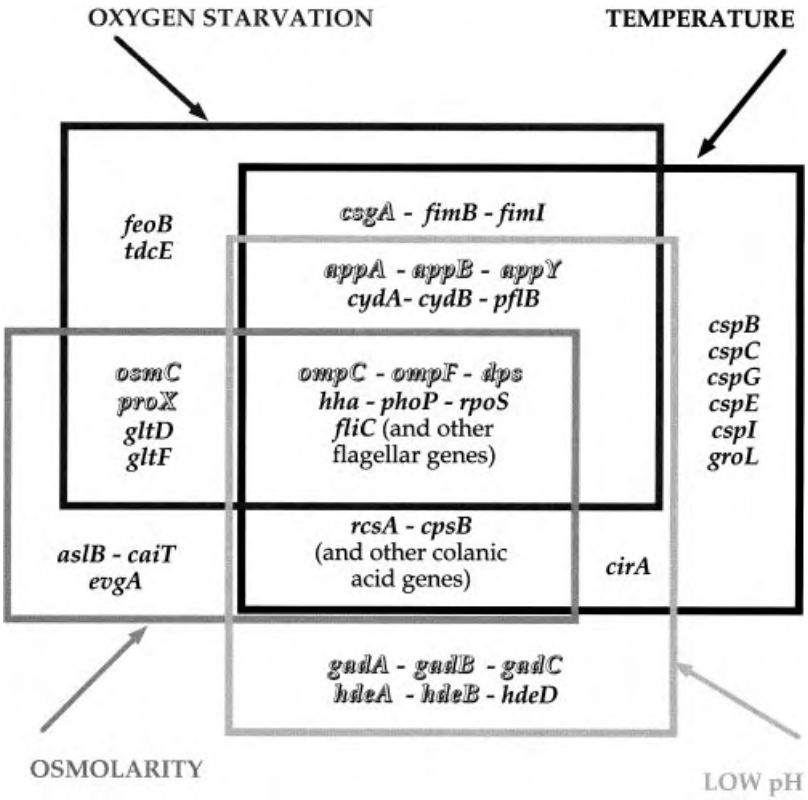

Fig. 6. H-NS regulon genes and their regulation by environmental parameters. Genes of known function that were found to be either directly or indirectly controlled by H-NS on two-dimensional gels (Fig. 1) and/or macroarrays (Table 1) were clustered according to the conditions known to modulate their expression. Rectangles represent genes belonging to oxygen, temperature, osmolarity and pH regulon respectively. Genes written in shaded are those identified previously as RpoS-regulated genes.

is therefore tempting to speculate that most of these gene products of unknown function (Table 1) also play a role in bacterial adaptation to different environmental conditions. In particular, YadN, YaiP, YagX and YcbQ, which are similar to fimbriae or adhesins, could be involved in bacterial colonization and/or virulence.

Among the targets of H-NS, many genes (for example, *ompC*, *gnd*, *proX*, *fliC*, *yciF* and *yeeN*) encoding proteins that were differentially accumulated in the *hns* strain (Fig. 1) showed, as expected, altered transcript levels on DNA arrays (Table 1). In contrast, some proteins, such as CbpA, were preferentially accumulated in the *hns* strain, but no significant difference in their transcript level was observed between wild-type and mutant strains. Moreover, several genes and proteins known to present a differential rate of expression or synthesis, respectively, in an *hns* mutant (Atlung and Ingmer, 1997; Laurent-Winter *et al*., 1997) did not show any difference in transcript level in wild-type or mutant strains on DNA arrays (Table 1). Several hypotheses may explain these discrepancies. First, some H-NS-regulated genes (for example, *cadBA and adiA*) are preferentially expressed in growth conditions that differ greatly from those used in the present study (aerobiosis and minimal medium supplemented with glucose). Similarly, these growth conditions could explain the lack of induction of the β-glucoside metabolism operon, *bglGFB*, in the *hns* mutant strain. Moreover, the transcript level of some genes, such as *yeiP*, was largely below the background level (data not shown), suggesting a low expression level and/or a rapid degradation of their corresponding mRNA. Finally, H-NS may affect the preferential accumulation of some proteins by a post-transcriptional mechanism, as observed recently for YceI: the lack of H-NS resulted in a minor effect on *yceI* expression (F. Hommais, unpublished), which is inconsistent with the great level of accumulation of YceI observed in the mutant strain on two-dimensional gel (Laurent-Winter *et al*., 1997).

H-NS post-transcriptionally regulates the synthesis and stability of the stationary sigma factor RpoS (Barth *et al*., 1995; Yamashino *et al*., 1995). An increase in the amount of *rpoS* mRNA was observed in the *hns* strain on DNA arrays (Table 1), suggesting that H-NS may also directly or indirectly regulate this gene at the transcriptional level and/or mRNA stability. We observed recently that the accumulation of a subset of H-NS-regulated gene products (GadA, Slp and HdeB) on two-dimensional gels is also controlled by RpoS and/or entry into stationary phase (C. Laurent-Winter, unpublished results). Strikingly, their genes showed the greatest increase in transcript level, between the *hns* mutant BE1410 and the wild-type FB8, ranging from a 23-fold to a 155-fold induction (Table 1). This effect on gene expression could be, at least partly, mediated by an increase in *rpoS* mRNA

(Table 1) and/or RpoS concentration in the *hns* mutant and further supports the existence of numerous interactions between both regulatory systems (Hengge-Aronis, 1999). Furthermore, these observations suggest that several other genes that are regulated by H-NS (Table 1) are also controlled by RpoS (Fig. 6).

Several H-NS-regulated genes encode DNA-binding proteins (Table 1). An increase in the transcript level of several regulators from two-component systems (PhoP, EvgA and YedW) was observed in the *hns* strain. In such systems, the control of gene expression depends on the phosphorylation state of the regulatory protein (Stock *et al*., 1989). Our results suggest that H-NS could initiate a new kind of regulation by specifically modulating the expression level of those regulators. Moreover, the effect of H-NS on genes of the colanic acid operon could be mediated by the increased expression of their activator-encoding gene, *rcsA* (Ebel and Trempy, 1999), in the *hns* mutant (Table 1). This suggests that H-NS plays an indirect role in the regulation of many genes identified in this study and further supports a high position of H-NS in the regulatory hierarchy of bacterial physiology. In particular, we demonstrated here that the overexpression of *gadX* (formerly called *yhiX*), which encodes a putative AraC family regulator (Table 1), in a wild-type strain increased the expression of H-NS-regulated genes involved in the response to low pH. Moreover, this strain showed a similar level of survival to the *hns* mutant at low pH in the presence of glutamate. These results strongly support an important role for GadX in the control of the genes involved in acid stress resistance with glutamate.

A higher level of mRNA induction and a greater amount of protein accumulation was observed in the H-NS-deficient strain than in the wild type (Table 1 and Fig. 1) for GadA and GadB, two glutamate decarboxylases involved in the response to low pH with glutamate (Lin *et al*., 1995). Moreover, the transcript levels of several genes and operons, such as *ompC*, *ompF* and *proU*, which are involved in the bacterial response to high osmolarity (Kempf and Bremer, 1998), and the accumulation of their products were also affected by the *hns* mutation (Table 1 and Fig. 1). At high osmolarity, an increase in the intracellular glutamate concentration and a K^+ accumulation is required to produce potassium glutamate, the primary cell protector against high osmolarity (Kempf and Bremer, 1998). Potassium glutamate may be an internal signal for the onset of the second phase of osmoadaptation, the uptake of compatible solutes, such as glycine betaine, which requires the *ompC* and *proU* gene products. The response to both low pH and high osmolarity results in the consumption of glutamate, which could explain the increase in *gltD* mRNA levels (Table 1). These observations suggest that the *hns* mutation severely perturbs glutamate metabolism.

The lack of H-NS has usually been considered as a loss of function, such as loss of motility (Soutourina *et al*., 1999) or reduction in growth rate (Barth *et al*., 1995). Paradoxically, survival assays showed that an *hns* mutation can constitute an advantage for bacterial cells at high osmolarity or low pH. In addition to the genes directly involved in low pH and high osmolarity resistance, numerous H-NS-regulated genes are controlled by both stresses (Fig. 6). In particular, some of them, such as those encoding porins, flagellum components and colanic acid, are also controlled by oxygen starvation or high temperature (Shi *et al*., 1993; Whitfield and Roberts, 1999), which reflects the growth conditions frequently encountered by enterobacteria inside their host (Mahan *et al*., 1996). All these responses imply cation exchange which is required for the establishment of homeostasis (Booth, 1999). These results and the observation that the modification of membrane protein composition (Table 1) is usually associated with the adaptation of bacteria to stressful environmental conditions (Kadner, 1996) suggest a strong relationship between the control of gene expression by H-NS and the maintenance of intracellular homeostasis. Thus, an *hns* strain could constitute a unique model for identifying the genes that enable pathogens to survive better inside their hosts.

Experimental procedures

Bacterial strains and growth conditions

Escherichia coli FB8 (Bruni *et al*., 1977) and MG1655 (Bachmann, 1983) strains and their *hns* mutant derivatives, BE1410 (Laurent-Winter *et al*., 1997) and BE1816 (Soutourina *et al*., 1999), respectively, containing a Tn*5*seq1 transposon in the 20th codon of the *hns* coding sequence (E. Krin, unpublished), were used. Bacteria were grown at 37°C in M63 minimal medium (Miller, 1992) supplemented with 0.4% glucose.

Construction of an E. coli *genomic DNA library*

pDIA561 was constructed by replacing the fragment between the *Xba*I site and the *Sac*I site in pBCKS+ (Stratagene) with the pcDNA 2.1 polylinker that contained two *Bst*XI sites (Invitrogen). Genomic DNA was isolated from *E. coli* mutated in the *hns* and *stpA* genes and nebulized for 30 s at 1 bar. Fragments ranging from 1.5 to 4.5 kb were ethanol precipitated, filled in by T4 polymerase and linked to *Bst*XI adapters (Invitrogen). Restriction fragments and pDIA561 digested by *Bst*XI were ligated by T4 DNA ligase at 16°C for 15 h. The ligation mixture was introduced into XL1-Blue strain (Stratagene) by electrotransformation. About 60 000 independent clones were selected on LB plates containing 20 μg ml^{-1} chloramphenicol and pooled. Large-scale plasmid DNA isolation was carried out using the JETstar kit (Genomed). This library was used to transform FB8.

Construction of plasmids

To overexpress *yhiX*, its coding sequence was amplified by

PCR from genomic DNA using primers 5′-ATGGAATTCCAA TCACTACATGGGAATTG-3′ and 5′-CGGGATCCTATAATC TTATTCCTTC-3′. These primers introduced an *Eco*RI cloning site into its 5′ end and a *Bam*HI site in its 3′ end. The PCR product was inserted into the *Eco*RI and *Bam*HI sites of the pTRC99A (Pharmacia), thus resulting in pDIA570.

High osmotic resistance

Bacteria were grown overnight in K5 medium (Epstein and Kim, 1971) supplemented with 0.4% glucose, 0.1% casamino acids. Cells were diluted 1:1000 in fresh K5 medium supplemented with 0.4% glucose and 3 M NaCl for 1 h at 37°C and then plated on LB. Viable cells were counted after 16 h at 37°C.

Low pH resistance

Bacteria were grown overnight in M9 medium (Miller, 1992), pH 5.5, supplemented with 0.4% glucose, 0.1% casamino acids. Cells were diluted 1:1000 in M9 medium, pH 2.5, supplemented with 0.2% glucose, 0.012% amino acids (glutamate, arginine or lysine) (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.

Two-dimensional gel electrophoresis

Total protein extracts and two-dimensional gel electrophoresis were carried out as described previously (Laurent-Winter *et al*., 1997) with some modifications. Total protein was extracted from *E. coli* cells ($A_{600} = 0.6$), and 50 μg was loaded onto isoelectrofocusing (IEF) gels containing ampholines with pH ranging from 3.5 to 10 (Millipore). This optical density of the culture corresponds to a late-exponential phase in minimal medium for both strains, despite a slightly slower growth rate of the *hns* mutant. The second dimension was carried out in 12.5% acrylamide slab gels. Resolved proteins were detected by silver nitrate staining (Morrissey, 1981). The gels were analysed, and the spots were quantified on the MELANIE II software (Bio-Rad). Values were expressed as percentage volume, which is the percentage ratio between the volume of a single spot and the total volume of all the spots on a gel. Volume is a function of optical density and spot area, which takes into account the variability resulting from silver staining. Spots of interest were excised, digested in trypsin (Shevchenko *et al*., 1996), desalted using Zip Tips C18 (Millipore) and eluted with 50% acetonitrile. MALDI-TOF spectra of peptides were obtained with an STR-DE mass spectrometer (Perspective Biosystems) using 2,5-dihydroxybensoic acid as a matrix. The sample was loaded on the target by the dried droplet method. Spectra obtained for the whole protein were calibrated externally using the [M+ H+] ion from Des Arg Braddykin peptide (904.47) and ACTH (2465.13). Peptides from the autodigestion of tryptin were used for the internal calibration. Samples were analysed in the reflectron mode, with an accelerating voltage of 25 000 V, an extraction delay of 200 ns and an average of 250 scans. Proteins were identified by comparing the spectra with non-redundant databases (SWISSPROT, Trembl and GenBank) using the ProFound, PeptIdent and MS-Fit web servers (http://prowl.rockefeller.edu/cgi-bin/ProFound, http://www.expasy.ch/tools/peptident.html, http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm).

Handling of RNA

Bacterial cells were grown to $A_{600} = 0.6$ (see above), and 5 ml of bacterial culture was centrifuged at 4°C for 10 min at 6000 *g* and stored at −80°C to prevent RNA degradation. Cells were lysed, and RNA was extracted according to the manufacturer's recommendations (Sigma-GenoSys Biotechnologies) using phenol, pH 4.5, at 65°C. RNA was ethanol precipitated and redissolved in 420 μl of 10 mM Tris, 1 mM EDTA, pH 7.6 (TE). RNA was incubated in 3 mM $MgCl_2$ with 20 μl of DNase I RNase free (Roche) for 30 min at 37°C to remove genomic DNA. DNase I was removed by a phenol–chloroform extraction. Purified RNA was ethanol precipitated, redissolved in 100 μl of TE and quantified by measuring A_{260} and A_{280}. 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 of RNA following standard cDNA synthesis using [α-^{33}P]-dCTP (2000–3000 Ci mmol^{-1} from New England Nuclear) as recommended by Sigma–GenoSys Biotechnologies. To prevent any subsequent amplification, the AMV reverse transcriptase (25 U μl^{-1}; Roche) with RNase H activity was used for cDNA synthesis. Alternatively, hybridization probes were generated from 10 μg of RNA in a final volume of 120 μl by incubating 4 μl of *E. coli* labelling primers (Sigma–Genosys), 0.25 mM each dATP, dGTP and dTTP with 60 μCi of [α-^{33}P]-dCTP and 6 μl of AMV reverse transcriptase for 3 h at 42°C. Unincorporated nucleotides were removed from labelled cDNA by gel filtration through a G-25 Sephadex column (Roche).

Hybridization

Macroarrays were Panorama *E. coli* gene arrays obtained from Sigma–GenoSys Biotechnologies. Prehybridization and hybridization were carried out according to the manufacturer's recommendations with some modifications. Hybridization and washing steps were carried out as described by the manufacturer using SSPE solution (0.18 M NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA, pH 7.7). The arrays were prehybridized in 15 ml of hybridization solution (5× SSPE, 2% SDS, 1× Denhardt's reagent, 100 μg of sheared salmon sperm DNA ml^{-1}) in roller bottles and hybridized for 16 h in a hybridization oven with 10 ml of hybridization solution containing the entire cDNA probe. Blots were washed in 0.5× SSPE, 0.2% SDS, slightly dried on a Whatman paper, wrapped and sealed in a damp Saran 25 μm film (Dow). Blots were exposed to a PhosphorImager screen (Molecular Dynamics) for 22 h. The arrays were stripped with boiled buffer (10 mM Tris, pH 7.6, 1 mM EDTA, 1% SDS) four times for 15 min and stored at room temperature in a sealed plastic

bag with 20 ml of 0.5% SSPE, 0.2% SDS. A single array could be used up to 10 times.

Data analysis

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 the XDOTSREADER software (Cose) and analysed using an EXCEL spreadsheet. The accuracy and reproducibility of the results were analysed by comparing the distribution of intensity in each spot between duplicate dots and between independent hybridizations. In all experiments performed with 1 μg and 10 μg of mRNA, the results (Fig. 2) showed a high degree of correlation (> 0.80), in agreement with recent data (Richmond *et al.*, 1999; Hoheisel and Vingron, 2000). The background noise was calculated from the expression of dots without DNA and subtracted from the intensity of each dot. Dot intensity was normalized according to the mean value of the total intensities of all spots on each DNA array, which allowed direct comparison of the two strains. The *hns* mutant expression profiles were compared with those of the wild-type strain by calculating the consistency of differential expression across replicate hybridizations using the Wilcoxon signed rank test, a non-parametric statistical method contained in the STATVIEW 5.0.1 package. This tested the hypothesis that one of the paired variables is greater or less than the other variable regardless of the magnitude of the difference and is appropriate for the analysis of small samples.

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