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Gene expression profiling of the low-pH response in *Escherichia coli*

V. Šeputienė,
K. Sužiedėlis,
E. Sužiedėlienė*

Department of Biochemistry and Biophysics, Faculty of Natural Sciences, Vilnius University, M. K. Čiurlionio 21, LT-2009 Vilnius, Lithuania

Escherichia coli acid-inducible genes were identified by whole-genome expression profiling. The culture was grown in a supplemented minimal low-phosphate medium (LPM) to the mid-logarithmic phase and the pH was shifted from 7.0 to 4.5. A total of 48 genes were induced in cells incubated at pH 4.5 for 2 h. The known genes *asr*, *hdeA* and *hdeB*, which have been characterized to be associated with acid resistance, were significantly induced. The *asr* was found to be the most strongly induced gene under the growth conditions tested. The other acid-induced genes represented functional grouping categories including central intermediatry metabolism (*aslB, glnK, yiaQ, yjfW*), translation (*infC*), post-translation modification (*pepT, hycI*), ribosome modification (*rmf*) and cell division (*minE*). Interestingly, 27 acid-induced genes code for hypothetical proteins with unknown function and their specific roles in acid response remain to be elucidated.

Key words: *E. coli*, acid stress, macroarray

INTRODUCTION

Over the last ten years the ability of pathogenic microorganisms to adapt to acidic pH through the expression of a range of gene products [1, 2] has excited a new interest in bacterial responses to acid stress. All gastrointestinal organisms, both pathogens and commensal organisms, must have the capacity to survive exposure to acidic environment (pH ~2.0) present in the stomach [3]. For any organism to survive a rapid shift to acidic pH there must exist protective mechanisms that allow bacterial cell surface structures to remain functional and cytoplasmic proteins and DNA to be protected or repaired.

Studies on acid stress response and survival strategies of enteric bacteria have evolved a range of complex mechanisms, which use different regulatory structures and genetic components required for survival and virulence. [4–6]. A variety of bacterial acid-inducible amino acid decarboxylase-based systems, which represent the best investigated examples, illustrates this complexity [7–9]. Decarboxylase-based systems consume protons that leak into cell during acid stress by decarboxylation of amino acids (glutamate, lysine, arginine and others), which are transported into the cell in exchange for their respective decarboxylated products [7, 8, 10, 11]. Decarboxylase systems provide the highest level of acid resistance [8, 12], allowing cells to survive extremely low pH challenges (pH 2.0) and pathogenic microorganisms to pass through gastrointestinal tract [4]. However, there are open questions regarding the molecular mechanisms of how amino acid inducible decarboxylase systems protect bacteria against strong acidity [8]. Moreover, the genetic and molecular components of another bacterial stress response and protection phenomenon known as acid tolerance response (ATR) are even less characterized [4, 13]. Exposure of enteric bacteria to moderate acidity (pH 4.5) induces a number of specific acid shock proteins, presumably belonging to different cellular systems, which confer efficient survival to a subsequent exposure to extreme acidic environments (pH 2.0) [14–17]. Most of these proteins are not characterized yet and their function in acid response is unknown [4, 16].

Functional genomic provides a comprehensive approach to identification of additional genes involved in acid response and thus allows to get more information on coordinated response to stress. In this study, *E. coli* was acid-shocked (pH 4.5) and the gene expression profiles of stressed cells were compared to those of cells grown at pH 7.0.

MATERIALS AND METHODS

Materials, bacterial strains and culture conditions. DNA macroarrays on nylon membranes, a kit for first-strand ^{33}P-labeled cDNA synthesis by using

* Corresponding author. E-mail: edita.suziedeliene@gf.vu.lt

AMV Reverse Transcriptase and hybridization solution were purchased from Sigma-Genosys Biotechnologies. The *E. coli* strain N2212 Rnase⁻(*rna*) *met relA ssrA*::*Cm* [18] used for genomic expression analysis was grown in a low-phosphate-glucose-salts medium LPM (50 mM Tris, 20 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 240 µM $MgCl_2$, 18.5 µM $CaCl_2$, 6.5 µM $FeCl_3$, 0.2% glucose, 0.6% Bacto Peptone) [19], buffered with MOPS (0.1 M pH 7.0). The overnight culture was diluted 1000-fold in LPM pH 7.0 and grown at 37 °C with rotary aeration to an optical density A_{590} of 0.5 (mid-logarithmic phase). Then half of the culture was harvested (control, pH 7.0). The pH of the residual culture was shifted from pH 7.0 to pH 4.5 (sample, pH 4.5) and incubation with rotary aeration at 37 °C prolonged for 2 h before harvesting.

Analysis of gene expression by using *E. coli* gene arrays. DNA macroarrays on nylon membranes (Sigma-Genosys Biotechnologies) containing 4290 ORF of *E. coli* printed in duplicate were used. Total RNA extraction and DNaseI (Sigma) treatment were performed according to manufacturers' recommendations. The C-terminal primer set (4290 ORF specific C-terminal primers (Sigma-Genosys Biotechnologies)) were used to generate the hybridization probe in a standard first-strand ^{33}P-labeled cDNA synthesis and the hybridization and washing steps were carried out according to manufacturer's recommendations (Sigma-Genosys Biotechnologies). The blots were exposed to a PhosphorImager screen (Molecular Dynamics) for 48 h. The exposed screens were scanned with a pixel size of 80 µm on a PhosphorImager (Molecular Dynamics). The resulting *.tif data files were analyzed by determining the pixel intensity for each spot in the array by using Array Vision software (Amersham Pharmacia). The software subtracted background automatically by using the local background for each spot subtraction method. The intensity of each spot was expressed as a percentage of the total of intensities of all the spots on the DNA array, which allowed direct comparison of the two conditions by normalizing with regard to specific activity of the probes used. The relative transcript levels were compared by determining the ratio of the corresponding intensities of each pair of ORF on two blots representing different growth conditions. A 2.5-fold expression ratio was considered to indicate a significantly higher expression.

RESULTS AND DISCUSSION

To identify the genes inducible by acid shock, we grew *E. coli* in LPM medium pH 7.0, shifted the pH to 4.5 and compared the gene expression profiles of cells grown at pH 7.0 to cells stressed at pH 4.5. RNA samples from pH 7.0 and 4.5 cultures were labeled and hybridized to the membranes of DNA arrays (Sigma-Genosys) (Figure). Genes with a significant differential expression had to meet the criterion – a of a 2.5-fold expression ratio. In this study, 47 *E. coli* genes were found expressed at significantly higher levels at pH 4.5 when compared to those of the control cells incubated at pH 7.0 (Table) and 48 genes were expressed at significantly higher levels at pH 7.0. Since we were primarily interested in genes induced by acid, we did not give any further consideration to genes repressed by acid.

The annotations for the 48 genes and the assigned functional groups listed in Table are given according to NCBI database. Twenty-seven acid-induced genes code for hypothetical proteins with unknown function. *asr*, *hdeB* and *hdeA* genes were found to be most significantly acid-induced with the estimated induction ratio 29.4, 10.7 and 4.5, respectively. The acid-induced expression of the genes listed above has been reported previously [20, 17]. *asr, hdeAB* were shown to be required for *E. coli*

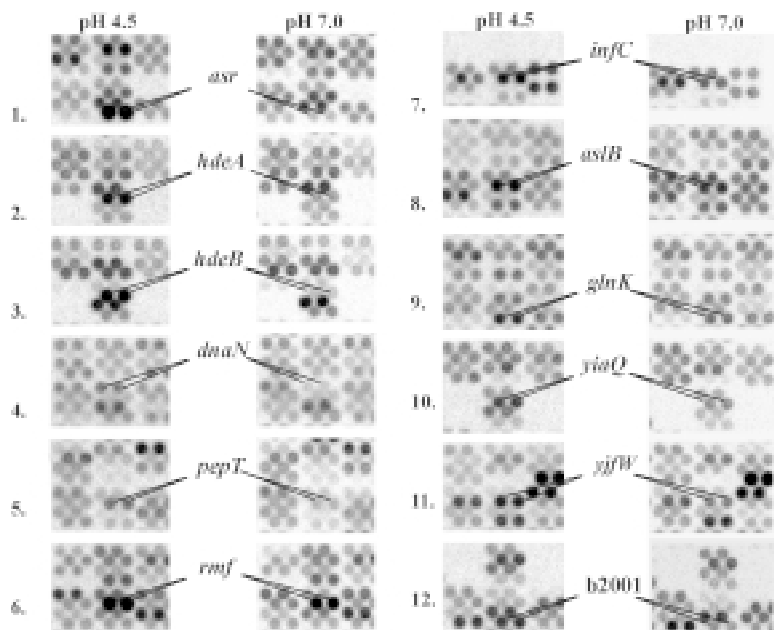

Figure. Fragments of DNA arrays of the entire set (4290 open reading frames in replica) of *E. coli* genes. Macroarrays were hybridized with cDNA probes generated from RNA extracted from cells grown in LPM pH 7.0 and acid-stressed at pH 4.5 for 2 h (indicated on the top). Macroarrays were scanned as described in Materials and Methods. Array Vision software was used for data analysis. Positions of the genes whose expression was induced upon acid stress are indicated

Table. **Expression ratios of acid-induced gene comparisons (pH 4.5 *vs*. 7.0)**

Gene	Gene product	Functional group	Induction ratio[a]
aslB	putative arylsulfatase regulatory protein	central intermediary metabolism	2.8
asr	periplasmic protein, acid resistance phenotype	unknown	29.4
b0395	hypothetical protein	unknown	3.0
b0984	hypothetical protein	unknown	4.0
b1005	hypothetical protein	unknown	4.0
b1191	hypothetical protein	unknown	4.1
b1273	hypothetical protein	unknown	2.4
b1312	hypothetical protein	unknown	2.9
b1436	hypothetical protein	unknown	3.3
b1445	hypothetical protein	unknown	3.2
b1582	hypothetical protein	unknown	2.8
b1629	hypothetical protein	unknown	6.1
b1731	hypothetical protein	unknown	2.8
b1791	hypothetical protein	unknown	2.7
b1957	hypothetical protein	unknown	3.1
b2001	hypothetical protein	unknown	6.1
b2229	hypothetical protein	unknown	2.7
b2266	hypothetical protein	unknown	3.5
b2790	hypothetical protein	unknown	3.5
b2791	hypothetical protein	unknown	4.7
b3010	hypothetical protein	unknown	3.1
dnaN	DNA polymerase III beta-subunit	DNA replication, recombination, modification	3.3
glnK	nitrogen regulatory protein P-II	central intermediary metabolism	2.8
glpT	glycerol-3-phosphatase transporter	transport and binding protein	3.0
hdeA	periplasmic chaperone, acid resistance phenotype	chaperone	4.5
hdeB	periplasmic protein	cell envelope	10.7
hycI	cognate endoprotease	degradation of proteins, peptides	3.3
yacG	hypothetical Zn binding protein	unknown	2.9
ydcH	hypothetical 6.5 kD protein in rimL 5'region	unknown	4.0
ydjB	hypothetical 23.4 kD protein in ansA 3'region	unknown	3.4
yebA	hypothetical 46.7 kD protein in msbB-ruvB intergenic region	unknown	3.2
yeiA	putative oxidoreductase	unknown	2.9
yfeG	putative ARAC-type regulatory protein	putative regulatory protein	2.9
yheD	putative general secretion pathway protein b	unknown	3.1
yhhI	putative receptor	putative regulatory protein	2.8
yi21_3	insertion sequence IS2 hypothetical protein	transposon-related function	3.2
yj21_4	insertion element IS2 hypothetical 13.4 kD protein	transposon-related function	2.7
yiaQ	probable 3-hexulose 6-phosphate synthase	central intermediary metabolism:	3.2
yiiU	hypothetical 9.6 kD protein in glpf-hslu intergenic region	unknown	3.8
yjbB	putative alpha helix protein	structural proteins	3.4
yjfW	putative hexulose-6-phosphate isomerase	central intermediary metabolism:	2.7
yjgG	hypothetical 12.8 kD protein in pyrL-argI intergenic region	unknown	2.9
infC	initiation factor IF-3	translation, post-translational modification	2.9
minE	cell division topological specificity factor	cell division	2.9
narY	respiratory nitrate reductase 2 beta chain	energy metabolism	3.4
pepT	aminotripeptidase	translation, post-translational modification	2.8
rmf	ribosome modulation factor (protein E)	ribosomes – maturation and modification	2.7
tmk	thymidylate kinase	nucleotide biosynthesis and metabolism	3.3

[a] – induction ratio of normalized duplicate spot intensities at pH 4.5 *vs*. pH 7.0.

growth and survival in acidic environment [11, 21, 22].

The *asr* was found to be the most significantly acid-induced bacterial gene at a moderate acidity in presently used supplemented minimal medium such as LPM (Table). A recently published study of gene expression profiling of *E. coli* grown in acidified (pH 4.5 and pH 5.5) minimal medium revealed the strongest expression of *asr* as well [17]. It was shown earlier that the transcription level of *asr* depends on medium composition: transcription is significantly inhibited in a complex medium if compared to LPM or minimal medium [20]. The gene encodes a 102 a.a. periplasmic protein of unknown function [20]. It has been recently demonstrated that *asr* is required for bacterial growth at a moderate acidity (pH 4.5) and for the induction of acid tolerance response (ATR) at a moderate pH [21]. The ATR phenomenon was observed in a broad range of bacteria [23]: if cells are left to adapt at a moderate pH (pH 5.0–4.0), numerous acid shock proteins are synthesized and these are needed to efficiently protect the cells from a subsequent treatment with othervise lethal levels of acid (pH 2.0–3.0) [24]. It has been proposed [17, 21] that highly basic Asr protein (pI 10.5) might serve as a proton sink in the periplasm by sequestering protons and protecting the essential proteins from denaturation. Tucker et al. showed that the transcription of genes encoding a small basic protein, YeaQ (82 a.a.. pI 11.1) and small acidic proteins was induced upon acid stress [17]. These results support the importance of small basic and acidic proteins, which could act as acid stress chaperons.

The *E. coli hdeAB* operon encodes two periplasmic proteins HdeA (11.8 kDa) and HdeB (12.5 kDa) [25]. There is genetic evidence that HdeA supports acid resistance in *E. coli*: survival at pH 2.0 of the *hdeA* mutant is severely compromised [22]. The mutation of the *hdeA* homologue from *Shigella flexneri* made this microorganism exquisitely acid-sensitive as well [11]. Functional *in vitro* studies demonstrated that *E. coli* HdeA is activated by a dimer-to-monomer transition at acidic pH, leading to suppression of aggregation of acid-denatured proteins. HdeA may support chaperon-like functions under acidic conditions [22]. HdeB is thought to form a heterocomplex with HdeA [11]. Other studies [26, 27] revealed the transcription of *hdeAB* in *E. coli* to be upregulated by acetate treatment, showing an overlap of the mechanisms that confer resistance to acid pH and to short-chain fatty acids (SCFA) such as acetate. SCFA equilibrates across the cytoplasmic membrane and lower internal pH even when the external pH is neutral [28].

In this study, we found that four genes (*yiaQ, yjfW, aslB, glnK*) encoding the components of central intermediary metabolism are acid-induced. The *yiaQ,* and *yjfW* genes encode putative 3-hexulose 6-phosphate synthase and putative hexulose-6-phosphate isomerase, respectively. No experimental data to show the expression regulation and function of the *E. coli* genes listed above have been reported yet.

The *aslB* gene encodes a putative regulator of the *E. coli* arylsulfatase gene *aslA*. Homologous systems are present in *Klebsiella pneumoniae* (AtsB) and *Klebsiella aerogenes* (AtsB). *K. pneumoniae* AtsB is involved in posttranslational activation of the cognate arylsulfatase AtsA by formylglycine modification [29] and *K. aerogenes* AtsB controls arylsulfatase AtsA [30]. The *E. coli* K1 *aslA* mutant was found to have a significantly decreased ability to invade brain microvascular endothelial cells compared to the wild type, which causes neonatal meningitis, a devastating disease with high mortality [31]. Low pH has been found as an important signal to the bacterium informing that it has entered a potential host environment, triggering the induction of many virulence genes [32–36]. Our observation that acidification induces expression of putative activator for *aslA*, which contributes to the invasion process of pathogenic *E. coli* may represent an example of acid-regulated virulence gene expression.

glnK encodes a small trimeric signal transduction protein that together with its paralogue PII protein regulates the activity of the enzymes and the expression of the genes (Ntr) involved in nitrogen assimilation during nitrogen starvation [37–39]. The expression of *glnK* was shown to be dependent on nitrogen starvation [38]. No experimental data to prove acid-regulated expression of *glnK* have been observed before. The activity regulation of glutamine synthetase, which converts glutamate into glutamine, is the central target of the signal transducers GlnK and PII [40]. GlnK and PII can form mixed oligomers, which could down-tune the activity of PII required for activation of glutamine synthase [39]. Glutamate plays a crucial role in *E. coli* acid resistance, which requires a glutamate-dependent decarboxylases system [10]. Thus, acid induction of GlnK regulator protein could be involved in the modulation of glutamine synthase activity in order to maintain the level of glutamate necessary for decarboxylation reaction.

Four genes (*rmf, infC, pepT, hydC*) involved in translation, ribosome maturation and protein modification were found to be acid-induced. *rmf* encodes the ribosome modulation factor RMF, a small basic protein of 55 amino acid residues, which is one of the stationary-phase-specific gene products [41]. RMF was shown to bind to the 50S ribosomal subunit and di-

rect the dimerization of 70S ribosomes into the 100S form, which has no translational activity [42]. By this mechanism the excess of unused ribosomes is converted into translationally inactive 100S dimers upon entry into the stationary phase. 100S ribosomal dimers are the storage form for ribosomes in stationary phase: after transferring the stationary cells to a fresh medium the 100S particles release the RMF and dissociate to yield functional 70S ribosomes [42, 43]. It has been reported that under slow growth conditions, *rmf* is expressed even in exponential phase and there is an inverse relationship between the expression of *rmf* and the cell growth rate [43]. Acidification of the growth medium to pH 4.5 causes a significant drop in *E. coli* growth [21]. Thus, acid-inducible transcription of *rmf* suggests that RMF could be involved in the induction of ribosome dimerization and translation arrest in *E. coli* cells upon acid stress conditions as well.

pepT encodes aminotripeptidase T, which is able to remove N-terminal amino acid from various tripeptides [44]. This gene was shown to be more highly expressed in biofilm cells than in free-living bacteria and to be highly induced in the late exponential growth phase [45]. Acid-induced upregulation of *hycI* encoding the endoprotease was detected as well. HycI (17 kDa) protease is involved in the maturation process of the large subunit of *E. coli* hydrogenase HycE. HycI catalyses the C-terminal proteolytic cleavage of the large subunit of the HycE [46]. The increased expression of *hycI* and *pep*T mRNA, observed in our study, might indicate that various proteolytic systems are activated in *E. coli* upon acid shift, possibly as a response to a stress-induced protein denaturation.

Interestingly, the expression of *dnaN* encoding the β subunit of DNA polymerase III holoenzyme, the *E. coli* chromosomal replicase, was induced upon acid stress (Table). The β subunit is a sliding DNA clamp responsible for tethering the polymerase to DNA and endowing it with high processivity [47]. In exponentially growing cells, *dnaN* is expressed predominantly from the *dnaANrecF* operon promoters [48]. However, it has been found that *dnaN* expression drastically changes in stationary phase independently of the *dnaA* regulatory region, and the synthesis of the β subunit increases in exponentially growing cells in response to moderate hyperosmotic conditions that only slightly reduce the growth rate [49]. DNA polymerase III holoenzyme is also an important component of DNA repair machinery and interacts with the other proteins involved in DNA repair, such as DNA polymerase II [47]. Exposure to an acidic environment has been shown to increase the amount of DNA damage: *Salmonella* and *E. coli* DNA sustain both depurination and methylation damage, *Streptococcus mutans* DNA undergoes degradation [50–52]. Thus, induction of β subunit expression by acid stress could be associated with the DNA repair process, which is important for efficient cell survival under acidic conditions [51, 53, 54].

Macroarray analysis revealed the acid-induced upregulation of *minE*, which encodes the cell division topological specificity factor. It is part of the protein system MinCDE, which regulates accurate cell division in *E. coli* [55]. The function of MinE is to localize correctly the MinCD proteins, which inhibit the assembly of the cell division ring [56]. *E. coli* cells contain only ~200 molecules of MinE per cell [57] and overexpression (400 molecules/cell) of *minE* leads to the formation of anucleate minicells [55, 57]. There are no experimental data concerning the transcription regulation of *minE*. The acid-induction of *minE* expression shows that the fidelity of midcell septum formation under acid stress could be reduced, leading to abnormal cell division and minicell phenotype.

The remaining acid-inducible genes (*yfeG, yhhI, glpT, yi21_3, yi21_4, infC, yjbB, narY, tmk*) have not been shown to possess an obvious role in acid resistance and therefore pose interesting questions.

Overall, our results yield an insight into possible acid stress induced changes in gene regulation that coordinate the processes such as acid-inducible resistance mechanisms, cell translation machine, metabolism and cell division. Tucker et al. identified a number of genes, including five functional categories (8 genes involved in metabolism, 9 associated with cell envelope structures or modification, 2 encoding chaperones, 6 regulatory genes), which were upregulated in *E. coli* grown on acidified (pH 4.5–5.5) glucose minimal medium if compared to those of cells grown at pH 7.4 [17]. Notably, the set of genes identified in this study is completely different from Tucker's study, except for *asr*, *hdeA* and *hdeB*, which have been characterized to be associated with acid resistance. These data extend the complexity of acid response in *E. coli*: the large number of acid-stress-induced genes may reflect the need for different acid-defense systems, depending on conditions to which cells are exposed, *e.g.*, the level of acidity, growth phase, the availability of nutrients or O_2 supply. In this study, 27 acid-induced genes that code for hypothetical proteins with unknown functions were identified, and their specific roles in acid resistance remain to be elucidated.

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Vaida Šeputienė, Kęstutis Sužiedėlis, Edita Sužiedėlienė

Escherichia coli VISO GENOMO EKSPRESIJOS ANALIZĖ RŪGŠTINIO STRESO SĄLYGOMIS

S a n t r a u k a

Escherichia coli genų raiškos pokyčiai rūgštinio streso sąlygomis buvo įvertinti panaudojant DNR gardeles. Logaritminės fazės ląstelės, augintos LPM pH 7,0 terpėje, 2 valandas buvo veiktos rūgštiniu stresu pH 4,5. Nustatyta, kad 48 genų transkripcija buvo indukuota ląstelėse, veiktose rūgštiniu stresu pH 4,5, lyginant su ląstelėmis, augusiomis terpėje, kurios pH 7,0. *asr*, *hdeA, hdeB* genų ekspresija buvo stipriausiai indukuojama rūgštinio streso metu. Nustatyta, kad *asr* geno transkripcijos pokytis yra didžiausias rūgštinį stresą patyrusiose ląstelėse. Rūgštinio streso metu buvo indukuojami centrinio metabolizmo (*aslB, glnK, yiaQ, yjfW*), transliacijos (*infC*), baltymų potransliacinės modifikacijos (*pepT, hycI*), ribosomų modifikacijos (*rmf*), ląstelės dalijimosi (*minE*) funkcinių grupių genai. Rūgštinio 27 streso metu indukuojami genai koduoja nežinomos funkcijos hipotetinius baltymus ir jų reikšmė bakterijų rūgštinio streso atsake dar nėra ištirta.