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Analysis of acid-induced *asr* gene promoter of *Enterobacteriaceae*

V. Ðeputienë,
K. Suþiedëlis,
E. Suþiedëlienë*

Department of Biochemistry and Biophysics, Vilnius University, Vilnius LT-2009, Lithuania

Enterobacteria in response to an external acidification strongly induce the transcription of *asr* gene. In this study mutational analysis of *E. coli asr* promoter has been performed to locate regions important for acid-triggered transcriptional activation. Our study revealed that deletions within the predicted *pho* box located at the –21/–38 region of the *asr* promoter did not absolutely abolish acid-induced transcription indicating the existence of additional cis-regulatory elements involved in transcription regulation. Moreover, the consecutive deletions of upstream sequences located in the –40/–70 region were found important for the acid-triggered transcriptional activation of *asr*. A comparative sequence analysis of the promoter region of *asr* gene homologues of other enterobacteria demonstrated a high degree of conservation suggesting a common environmental stress-induced regulatory pathway.

Key words: acid stress, *Enterobacteriaceae*, *E. coli asr* promoter, mutational analysis

INTRODUCTION

In order to survive and adapt to changing environmental conditions, microorganisms have developed molecular regulatory networks that modulate gene expression in response to external signals [1]. Transcriptional analysis has demonstrated that operation of these networks depends on the physiological status of the cell and growth medium composition [2]. Multiple regulatory factors have been found to be involved in the transcription control of acid-induced genes [3, 4].

Acid shift of growth medium from pH 7.0 to a pH 5.0–4.0 induces *asr* mRNA in *Escherichia coli* [5] and in other enterobacteria [6]. The *asr* gene is required for bacterial growth at moderate (pH 4.5–4.6) acidity in a supplemented minimal LPM medium and for the induction of acid tolerance in log phase cells, which supports the survival in extreme acid (pH 2.0) [6]. The *E. coli* Asr protein has been shown to be transported into the periplasm during acid stress and processed to an 8 kDa polypeptide by a two-step cleavage [6]. The physiological function of Asr protein is presently unknown.

*Corresponding author.
Mailing address: Department of Biochemistry and Biophysics, Faculty of Natural Sciences, Vilnius University, M. K. Èiurlionio 21, Vilnius LT-2009, Lithuania.
E-mail: edita.suziedeliene@gf.vu.lt

asr mRNA is a long-lived and one of the most abundant RNA messages in *E. coli* cells at maximal levels of induction [5]. This was confirmed by mRNA expression profiling of *E. coli* cells grown in a supplemented minimal medium and subjected to acid shock [7]. Search for the trans-acting regulatory components responsible for *asr* transcription revealed that the *E. coli* PhoBPhoR regulatory system, alternative σ^s factor and global regulator H-NS are involved in the transcriptional control of *asr* [5, 6, 8, 9]. In the current study mutational analysis of the *E. coli asr* promoter region was performed to search for possible regulatory cis-sequences important for acid-triggered transcription regulation of *asr* gene. Transcriptional analysis of *asr* promoter mutants as well as a comparative analysis of promoter DNA sequences of *asr* homologues of other enterobacteria identified a conservative region required for transcriptional activation of *asr* under acid shock conditions.

MATERIALS AND METHODS

Materials, bacterial strains and plasmids. All the materials listed below were purchased from Sigma, Merck and Roth. Kits for molecular biology were from AB Fermentas. All enzymes were used as recommended by the supplier. The bacterial strains used were HB101 [10], JE13 (N2212*asr*::kan) [5]. Plasmid pAS2 harbouring 1.3 kb *E. coli* DNA

fragment containing *asr* gene [5]) and derivatives of pAS2 with the *asr* promoter mutations (Fig. 1) were used in this study.

Media and growth conditions. Unless otherwise indicated, *E. coli* cells were grown in Luria–Bertani (LB) medium [11]. When necessary, antibiotics were added at the following concentrations: ampicillin 100 μg ml^{-1}, kanamycin 60 μg ml^{-1}, chloramphenicol 34 μg ml^{-1}. The cells were acid-shocked as follows: cells from overnight cultures were diluted 1:1000 with a low-phosphate-glucose-salts medium LPM [12], buffered with MOPS (0.1 M pH 7.0) and grown at 37 °C with rotary aeration to an optical density A_{590} of 0.5. Then the cells were resuspended in the same volume of LPM medium, adjusted by HCl to pH 4.5, and incubated at 37 °C with rotary aeration.

DNA and RNA manipulations, plasmid construction. Plasmid DNA and RNA preparation, restriction endonuclease digestion, DNA ligation, DNA and RNA agarose gel electrophoresis, Northern blot analysis, DNA labeling with ^{32}P, DNA sequencing were performed according to standard protocols [11]. The derivatives of pAS2 containing mutations and deletions of *asr* promoter DNA (Fig. 1) were introduced by PCR (the primers are listed in Table). The mutations were verified by DNA sequencing.

	SEQUENCE: *pho* box: ctgtcataaaactgtcat	LTR[a]
	-80 -70 -60 -50 -40 -30 -20	
pAS2	ttttattcagcgttttgtacatatcgttacacgctgaaaccaaccactcacggaagtctgccattc	
pAP	tttattcagcgtttgtacatatcgttacacgctgaaaccaaccactcacggaagtctaccattc	1x
p$^{\Delta}$I	tttattcagcgtttgtacatatcgttacacgctgaaaccaa----------gtctgccattc	4x
p$^{\Delta}$II	tttattcagcgtttgtacatatcgttacacgctgaaaccaaccactcacggaa---------tc	5x
p$^{\Delta}$P	tttattcagcgtttgtacatatcgttacacgctgaaaccaac----------ttc	5x
p$^{\Delta}$70	----------gtacatatcgttacacgctgaaaccaaccactcacggaagtctgccattc	1x
p$^{\Delta}$37	tttat----------cactcacggaagtctgccattc	30x
p$^{\Delta}$20	tttattcagcgtttgtacatat----------cactcacggaagtctgccattc	25x
p$^{\Delta}$21	tttat----------acacgctgaaaccaaccactcacggaagtctgccattc	9x
p$^{\Delta}$10	tttattcagcgtttgtaca----------cgctgaaaccaaccactcacggaagtctgccattc	10x
p$^{\Delta}$4	tttattcagcgtttgtacatat----acacgctgaaaccaaccactcacggaagtctgccattc	7x
p$^{\Delta}$1	tttattcagcgtttgtacatat-gttacacgctgaaaccaaccactcacggaagtctgccattc	6x

[a]- level of transcription reduction: induction ratio pAS2 *vs.* mutated plasmid.

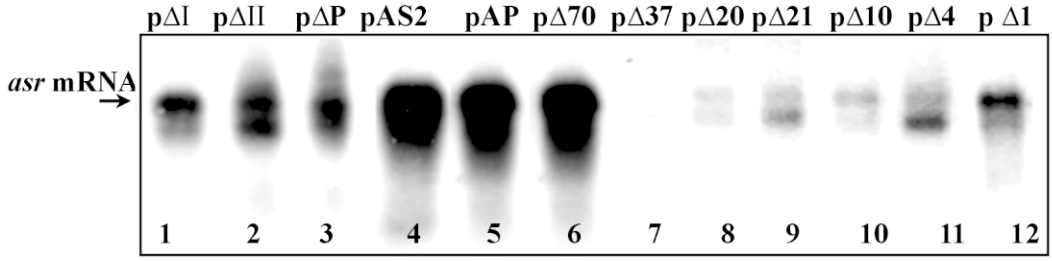

Fig. 1. Northern blot analysis of total cellular RNA isolated from strain JE13 carrying the plasmids with *asr* promoter DNA mutations. Cells were grown in LPM pH 7.0 to an optical density A_{590} of 0.5 and acid shocked as described in Materials and Methods for 2 h. 20 μg RNA was loaded into each lane. A ^{32}P labeled 0.47 kb *E. coli* DNA fragment from pAS2 containing *asr* gene was used as a hybridization probe. The obtained signals were quantified with Image-Quant software (Molecular Dynamics). The sequences of mutant plasmids carrying the deletions of *asr* promoter DNA are presented above: *pho* box, and PhoB binding sites are highlighted and underlined respectively, point mutation is indicated in bold, "–" corresponds to deleted region. The level of transcription reduction–induction ratio pAS2 *vs.* mutated plasmid is indicated on the right

RESULTS AND DISCUSSION

Mutational analysis of the predicted *pho* box. Previous DNA analysis of the *E. coli asr* promoter region revealed a 18-nt sequence CTCACGGAAGTCTGCCAT (Fig. 1, –21/–38 region, highlighted) [5] similar (12-nt from 18) to a consensus *pho* box (CTGTCATA(A/T)A(T/A)CTGTCAT) found in promoters of *E. coli pho* genes inducible by phosphate starvation [13]. The expression of *E. coli pho* genes comprising a Pho regulon is under the control of the two-component

Table. **Oligonucleotide primers used in this study. Mutated nucleotide is underlined**

Name	Sequence	Position	Constructed plasmid
VA10	5'-CTCACGGAAGTCTACCATTCC-3'	–40/–19	pAP
VA11	5'-TGGTTGGTTTCAGCGTGTAAC-3'	–61/–39	pAP, pΔI
VA4N	5'-TTCCGTGAGTGGTTGGTTTC-3'	–51/–30	pΔII
VA3N	5'-GTCTGCCATTCCCAGGGAT-3'	–31/–12	pΔI
VA5	5'-TTCCCAGGGATATAGTTATTT –3'	–20/+1	pΔII, pΔP
VA6	5'-GTTGGTTTCAGCGTGTAACG -3'	–61/–41	pΔP
VA9	5'-ATAAATTACAGCGTTGATAAT-3'	–100/–79	pΔ21, pΔ37
VA8	5'-ATATGTACAAACGCTGAATA-3'	–81/–61	pΔ20, pΔ10
VA7	5'-CACTCACGGAAGTCTGCCATT–3'	–41/–20	pΔ37, pΔ20, pΔ4, pΔ1
VAD1	5'-GTTACACGCTGAAACCAAC-3'	–59/–40	pΔ1
BG2	5'-ACACGCTGAAACCAA-3'	–56/–41	pΔ4, pΔ21, pΔ10

regulatory system PhoBPhoR [14]. It has been demonstrated that deletion of *E. coli phoBR* operon abolished the acid-induced transcription of *asr* gene, suggesting that the PhoBPhoR system might control the expression of *asr* [5]. Regulatory protein PhoB bound *asr* promoter DNA *in vitro* and could in this manner act as a positive activator of *asr* transcription [5]. In order to confirm the role of the predicted *pho* box *in vivo*, deletions of the first (pΔI, deleted region –30 / –41) and second (pΔII, deleted region –21 / –29) halves of the proposed PhoB binding site and deletion of the entire *pho* box (pΔP, deleted region –22 / –40) of *asr* were introduced (Fig. 1) and the activity of the *asr* mutant promoters was tested.

The plasmid pAS2 carrying the *E. coli* 1.3 kb DNA fragment with *asr* gene [5] was used for PCR site-directed mutagenesis. The effect of the resultant mutant *asr* promoter on the plasmid was tested in an acid-induced *asr* null mutant (strain JE13) by using Northern blot. Surprisingly, the deletions of the binding sites and the entire *pho* box resulted only in a 4–5-fold decrease of *asr* gene transcription level (Fig. 1, lanes 1, 2, 3 *vs.* 4), but did not absolutely abolish the acid-induced transcription of *asr* gene as was observed in *phoBR* mutant [5], suggesting that the predicted *pho* box or part of this box might be dispensable for the acid-induced transcriptional activation of *asr* gene. The partial reduction of *asr* expression could result due to deletion of RNA polymerase binding sites.

We next introduced a G→A substitution at the position –25 of the predicted second binding site of PhoB within the proposed *pho* box (pAP, Fig. 1). It has been shown previously that G→A substitution reduces activity of the PhoB-dependent *pstS* promoter 10-fold [15]. This point mutation had no effect on the transcription of *asr* (Fig. 1, lane 5 *vs.* 4), indicating that the predicted *pho* box did not represent a regulatory sequence required for acid-induced *asr* transcription.

Deletion analysis of *asr* promoter region upstream the proposed *pho* box. In order to identify potential cis-regulatory sites in the *asr* promoter, deletion analysis of the DNA region upstream the –40 position was performed. The consecutive promoter deletions (pΔ70, pΔ37, pΔ20, pΔ21, pΔ10, pΔ4, pΔ1 in Fig. 1) were introduced into the plasmid pAS2 by site-directed mutagenesis and tested for the transcriptional activity in *asr* null mutant (strain JE13). Deletion of the distal region upstream –70 did not influence the acid-induced activity of *asr* promoter (Fig. 1, lane 6 *vs.* 4), indicating that it contains no elements of *asr* transcription control. However, deletions close to the –60 position significantly decreased the activity of *asr* promoter (Fig. 1, lanes 7, 8, 9, 10, 11, 12). Notably, even the deletion of C at –60 position (pΔ1) resulted in a 6-fold reduction of promoter activity (Fig. 1, lane 12 *vs.* 4). Taken together, these observations indicate the existence of an acid regulatory region and a positive regulator which binds this area. The sequence of the *asr* promoter distal region upstream –38 does not closely resemble the PhoB binding site (*pho* box). Transcription factor binding sites in bacterial genomes are usually long, consisting of ~30 bases and variable. Often most of their regulatory sequences are carried in two conserved subregions, each about 6 bases in length [16], which contain the predominant contacts with the transcription factor. However, the region –40 / –70 of *asr* promoter does not resemble binding sites of other known transcription activators [17]. Nevertheless, a specific binding of PhoB protein to regions even poorly matching the *pho* box consensus resulting in the promoter activation has been demonstrated [18]. Numerous studies have indicated that several trans-acting regulators can contribute to selective activation of *E. coli* promoters in response to environmental stress [19–21] and the *asr* promoter might represent such an example.

Promoter sequence comparison of *asr* gene homologues of *Enterobacteriaceae*. To search for pos-

sible conservative cis-control elements of transcription regulation for *asr*, we decided to compare the promoter sequences of *asr* homologues from other *Enterobacteriaceae*. The *asr* gene homologues were identified in *Enterobacter cloacae, Klebsiella pneumoniae, Salmonella enterica* serovars Typhimurium, Typhi, Paratyphi and Enteritidis, *Yersinia pestis* and

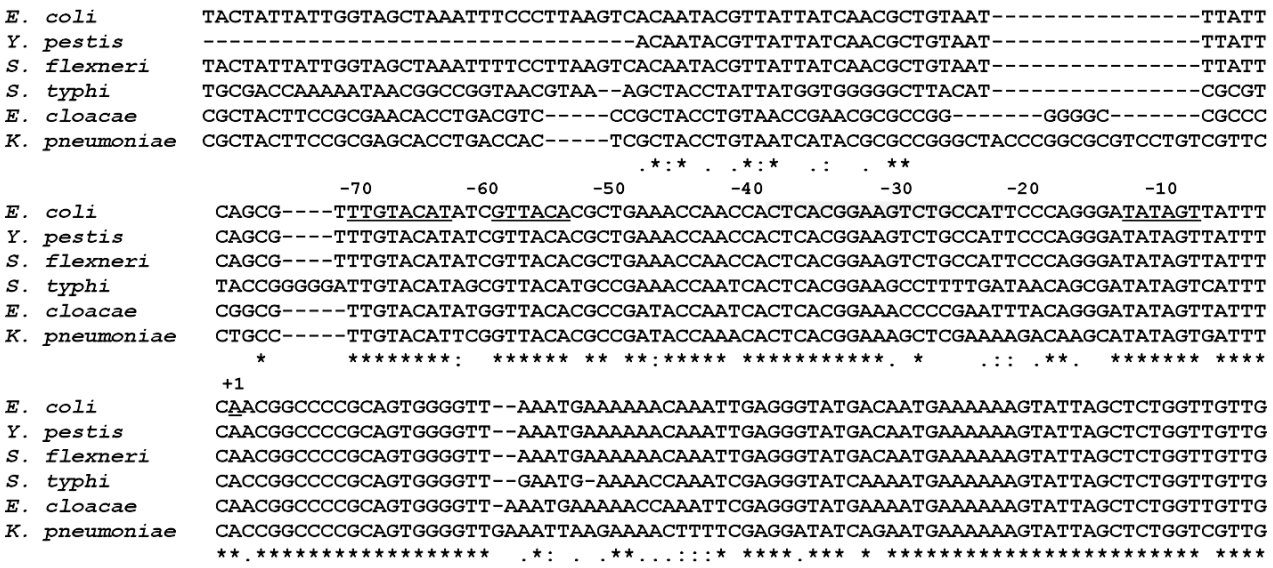

Fig. 2. Nucleotide sequence alignment of –140 / +150 promoter regions of *asr* homologues from *E. coli, Y. pestis, S. flexneri, S. enterica* serovar Typhi, *E. cloacae, K. pneumoniae*. Stars indicate identical nucleotides. The transcription start site +1, –10 box and –54/–70 region of *E. coli asr* are underlined, the proposed *pho* box of *E. coli* is highlighted

Shigella flexneri [6]. The expression of cloned *asr* homologues from *E. cloacae, S. typhimurium, K. pneumoniae* and *S. typhi* was tested in *E. coli* and found to be strongly acid-induced [6]. Nucleotide sequences alignment of the –140 / +150 promoter regions of cloned by us [6] and obtained from DNA data base (NCBI GenBank) *asr* gene homologues by the CLUSTAL W program [22] is presented in Fig. 2. The promoter regions of *asr* homologues exhibit a considerable homology up to –70 relative to the +1 transcriptional start. The transcriptional start site determined experimentally in *E. coli* as an adenine residue and predicted –10 box [5] are highly conservative in all examined enterobacteria. The sequences quickly diverge upstream position –70. Notably, the –21 / –30 region containing part of the proposed *pho* box (–21 / –38), which was dispensable for transcriptional activation of *asr* gene, does not exhibit any homology (Fig. 2). Of particular interest is the region –70 / –54 containing a 17 bp sequence TTGTACAT$(N)_3$GTTACA (Fig. 2, underlined). The mutational analysis of *E. coli asr* promoter described above showed that the deletions (pΔ21, pΔ10, pΔ4, pΔ1, Fig. 1) in this region significantly reduced the activity of *asr* promoter (Fig. 1, lanes 9, 10, 11, 12). Thus, this promoter region, which has been found to be important for acid-regulation of the *E. coli asr* gene, is highly conserved in all examined bacteria, indicating that it might represent a target for a complex regulatory circuit that controls gene expression in response to a low pH.

In conclusion, promoter deletion analysis located the region at position –70 to –40 important for the acid-triggered transcriptional activation of *asr*. Observations deduced from the alignment of promoter DNA of the *asr* homologues suggest a common expression regulation of *E. coli asr* counterparts in other enteric bacteria.

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ENTEROBACTERIACEAE ÐEIMOS BAKTERIJØ RÛGÐTINIO STRESO INDUKUOJAMO *ASR* GENO PROMOTORIAUS ANALIZË

Santrauka

Atsako á rûgðtiná stresà metu enterobakterijose yra indukuojama *asr* geno transkripcija. Siekiant nustatyti *asr* geno promotoriaus sritis, svarbias geno transkripcijos reguliacijai, buvo ávestos *E. coli asr* geno promotoriaus DNR mutacijos ir iðtirtas jø poveikis geno transkripcijai. Nustatyta, kad *asr* geno promotoriaus –21/–38 srityje spëjamos *pho* sekos delecijos tik ið dalies sumaþino þemo pH indukuojamà *asr* geno transkripcijà. Tai liudija, kad egzistuoja kiti cis-reguliaciniai elementai, svarbûs streso indukuojamai transkripcijai. Promotoriaus delecine analize nustatyta, kad *asr* geno promotoriaus sritis, esanti –40/–70 rajone, yra svarbi rûgðtinio streso indukuojamai geno transkripcijai. Atlikus enterobakterijø *asr* geno promotoriø DNR sekø palyginamàjà analizæ buvo nustatytos konservatyvios promotoriaus sritys. Tai leidþia manyti, kad enterobakterijose rûgðinio streso indukuojamo *asr* geno reguliacijoje dalyvauja konservatyvûs *Enterobacteriaceae* šeimos bakterijø transkripcijos reguliatoriai.