

## The Acid-Inducible *asr* Gene in *Escherichia coli*: Transcriptional Control by the *phoBR* Operon†

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*Escherichia coli* responds to external acidification (pH 4.0 to 5.0) by synthesizing a newly identified, ~450-nucleotide RNA component. At maximal levels of induction it is one of the most abundant small RNAs in the cell and is relatively stable bacterial RNA. The acid-inducible RNA was purified, and the gene encoding it, designated *asr* (for acid shock RNA), mapped at 35.98 min on the *E. coli* chromosome. Analysis of the *asr* DNA sequence revealed an open reading frame coding for a 111-amino-acid polypeptide with a deduced molecular mass of approximately 11.6 kDa. According to computer-assisted analysis, the predicted polypeptide contains a typical signal sequence of 30 amino acids and might represent either a periplasmic or an outer membrane protein. The *asr* gene cloned downstream from a T7 promoter was translated in vivo after transcription using a T7 RNA polymerase transcription system. Expression of a plasmid-encoded *asr::lacZ* fusion under a native *asr* promoter was reduced ~15-fold in a complex medium, such as Luria-Bertani medium, versus the minimal medium. Transcription of the chromosomal *asr* was abolished in the presence of a *phoB-phoR* (a two-component regulatory system, controlling the *pho* regulon inducible by phosphate starvation) deletion mutant. Acid-mediated induction of the *asr* gene in the  $\Delta(\textit{phoB-phoR})$  mutant strain was restored by introduction of the plasmid with cloned *phoB-phoR* genes. Primer extension analysis of the *asr* transcript revealed a region similar to the Pho box (the consensus sequence found in promoters transcriptionally activated by the PhoB protein) upstream from the determined transcription start. The *asr* promoter DNA region was demonstrated to bind PhoB protein in vitro. We discuss our results in terms of how bacteria might employ the *phoB-phoR* regulatory system to sense an external acidity and regulate transcription of the *asr* gene.

The ability to sense and respond to changing environmental conditions by turning on genetic regulatory systems has been recognized as an essential feature that enables many enteric bacteria to survive and successfully adapt to numerous stressful treatments (heat, osmolarity, starvation, radiation, anaerobiosis, etc.). Products of genes comprising these networks are involved in a broad range of cellular events, from receiving the initial signal to repairing the damages caused by stress (57).

In a variety of environments, low pH is a common condition with which many enteric bacteria, such as *Escherichia coli*, *Salmonella typhimurium*, and *Helicobacter pylori*, must cope. Enterobacteria respond to low pH by de novo synthesis of specific sets of proteins (23, 24) and the altered expression level of a number of genes, as has been demonstrated by gene-operon fusions (15, 22). Observations of the last few years have established that bacteria possess specific molecular mechanisms to respond and adapt to acid stress (for reviews, see references 5, 12, 45). The well known and investigated molecular systems responding to an environmental acidity are inducible amino acid decarboxylases. Their contribution to pH homeostasis (alkalinization of the cytoplasm by elimination of H<sup>+</sup> ions), when bacteria encounter acidity, has been demonstrated by the analysis of *E. coli* and *S. typhimurium cadBA*

operons (encoding lysine decarboxylase CadA and lysine/cadaverine antiporter CadB); the *E. coli* gene for arginine decarboxylase, *adiA*; and its regulator, *adiY* (39, 46, 55, 56). A similar role has been proposed for an *E. coli* glutamate decarboxylase and a putative glutamate/ $\gamma$ -amino butyrate antiporter (21). Whereas bacterial acid-inducible decarboxylases play an important role in pH homeostasis, survival and adaptation to extreme pH values, when constitutive pH homeostasis normally fails, require additional genetic systems. *E. coli* and *S. typhimurium* are able to tolerate severe acidity after exposure to a mild acid (14, 19). This complex acid tolerance response phenomenon in *S. typhimurium* has been intensively studied and shown to require the synthesis of over 50 acid shock proteins and to be growth phase regulated (4).

The expression of most low-pH-inducible genes identified so far in bacteria is also affected by other environmental signals (anaerobiosis, presence of nutrients, starvation, and specific host-produced factors), implying the intersection of different regulatory pathways and overlapping control of gene expression (12, 45). Here we report on the newly identified *E. coli* gene (*asr*) inducible by a low external pH. Acid-induced expression of *asr* is strongly reduced in complex Luria-Bertani (LB) medium compared to expression in minimal medium. Our experiments suggest that pH-triggered expression of *asr* may be regulated by the bacterial two-component regulatory system *phoB-phoR*, which controls the *E. coli pho* regulon inducible by phosphate starvation.

### MATERIALS AND METHODS

**Strains, plasmids, media, and growth conditions.** The bacterial strains, phages, and plasmids used in this study are listed in Table 1. *E. coli* cells were grown in LB medium (40). For in vivo labeling with <sup>32</sup>P and induction of RNA

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† Dedicated to David Apirion, whose death in 1992 was the loss of a devoted scientist.

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TABLE 1. *E. coli* strains, phages, and plasmids used in this study

Strain, plasmid, or phage	Genotype or description	Reference or source
<b>Strain</b>		
ANCK10	F <sup>-</sup> <i>leu lacY trp his argG strA ilv metA</i> (or <i>metB</i> ) <i>thi</i>	37
ANCH1	ANCK10 with $\Delta(\textit{phoB-phoR})$ Kan <sup>r</sup>	69
D10	RNase <sup>-</sup> ( <i>rna</i> ) <i>met relA</i>	28
HB101	F <sup>-</sup> <i>hsdS20</i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>leu supE44 ara14 galK2 lacY1 proA2 rpsL20 xyl-5 mtl-1 recA13 mcrB</i>	6
JE13	N2212 <i>asr::Kan</i>	This work
M8820	F <sup>-</sup> <i>araD139</i> $\Delta(\textit{ara-leu})7697$ $\Delta(\textit{proAB-argF-lacIPOZYA})XIII$ <i>rpsL</i>	9
M8820Mu cts	M8820 with Mu cts	8
MAL103	F <sup>-</sup> Mu dII <i>ara::</i> (Mu cts) $\Delta(\textit{proAB-argF-lacIPOZYA})XIII$ <i>rpsL</i>	9
MC4100	F <sup>-</sup> <i>araD139</i> $\Delta(\textit{argF-lac})205$ <i>flbB5301 ptsF25 relA1 rpsL150 deoC1</i>	8
N 2212	D10 <i>ssrA::Cm</i>	44
POI1734	MAL103 with Mu dII1734 (Kan <sup>r</sup> ) in place of Mu dII1734 (Ap <sup>r</sup> )	9
<b>Phage<sup>a</sup></b>		
$\lambda$ 311/E3F2	<i>asr</i> <sup>+</sup> ; position 35.98 min <sup>b</sup>	29
$\lambda$ 311::Kan	pAS4 recombined on $\lambda$ 311	This work
<b>Plasmid</b>		
pUC18/19	Cloning vectors	71
pUC4K	Vector carrying Kan <sup>r</sup> fragment	Pharmacia
pBR322	Cloning vector	6
pT7-6	Expression vector	59
pBC6 $\Delta$ PstI	<i>phoBR</i> operon in pUC18	65
pGP1-4	Vector with T7 RNA polymerase gene	59
pAS1	2.13-kb <i>Bgl</i> I fragment from $\lambda$ 311 cloned into pUC19	This work
pAS2	1.3-kb <i>Bgl</i> I- <i>Pvu</i> II fragment from $\lambda$ 311 cloned into pUC19	This work
pAS3	pAS1 without <i>Pst</i> I restriction site in MCS	This work
pAS4	1.3-kb kanamycin resistance gene from pUC4K cloned into <i>Pst</i> I site of pAS3	This work
pAS5	706-bp <i>Csp</i> 6I fragment from pAS2 cloned into <i>Sma</i> I site of pT7-6	This work
pAS6	1.3-kb <i>Bam</i> HI- <i>Eco</i> RI fragment from pAS2 cloned into pBR322	This work
pAS7	286-bp <i>Hind</i> III- <i>Bsp</i> 143II fragment from pAS5 cloned into pT7-6	This work
pAS8	pAS6 containing Mu dII1734(Kan <sup>r</sup> <i>lac</i> ) at position 127 in the <i>asr</i> DNA	This work
pVG6	2.0-kb <i>Mlu</i> I fragment with <i>phoB</i> gene from pBC6 $\Delta$ PstI cloned into <i>Sma</i> I site of pT7-6	This work
pVG11	2.0-kb <i>Mlu</i> I fragment with <i>phoB</i> gene from pBC6 $\Delta$ PstI cloned into <i>Sma</i> I site of pUC19	This work
pVG12	2.8-kb <i>Bpi</i> I- <i>Eco</i> RI fragment with <i>phoR</i> gene from pBC6 $\Delta$ PstI cloned into pUC18	This work

<sup>a</sup>  $\lambda$  311 corresponds to the numbering system of the miniset given by Kohara et al. (29).

<sup>b</sup> The chromosomal position of the cloned DNA fragment in minutes is given according to Médique et al. (38).

under acidic conditions, a low-phosphate-glucose-salts medium (LPM), supplemented with peptone (0.6 mg/ml), was used (28). LPM was buffered with MOPS (morpholinepropanesulfonic acid) (pH 7.0) or MES (morpholineethane sulfonic acid) (pH 5.0) to a final concentration of 0.1 M. For phosphate starvation analysis, MOPS minimal growth medium (MM), supplemented with 0.4% glucose, 0.01 M thiamine hydrochloride, and appropriate amino acids (20  $\mu$ g/ml), was used (42). Phosphate was added to the MM as K<sub>2</sub>HPO<sub>4</sub> to a final concentration of 0.01 mM (low P<sub>i</sub>) or 1 mM (high P<sub>i</sub>). To analyze the medium and phosphate starvation effect, cells were grown in the specified medium until the density of cultures reached approximately 10<sup>8</sup> cells ml<sup>-1</sup>. At this point, the pH of the growth medium was shifted down to 4.8, whereas that of the control cultures remained unchanged. After incubation for an additional 1 h, cells were assayed for  $\beta$ -galactosidase activity.

For solid media, the pH was adjusted prior to addition of agar (1.5%) and autoclaving. When necessary, the following antibiotics were added at the indicated concentrations: ampicillin, 100  $\mu$ g/ml; chloramphenicol, 34  $\mu$ g/ml; tetracycline, 20  $\mu$ g/ml; and kanamycin 60  $\mu$ g/ml.

**Growth and <sup>32</sup>P labeling of cells.** Cells were cultured overnight in LB medium at 30°C and inoculated the next morning into the LPM (usually 1 ml of the medium). When the cultures had reached an A<sub>560</sub> of 0.5 to 0.7, the pH was shifted to pH 4.8 to 5.0 by adding several drops of sterile 0.55 N HCl. After 30 min of incubation at 30°C, 50  $\mu$ Ci of H<sub>3</sub>PO<sub>4</sub> (carrier free; ICN Radiochemicals)/ml added and incubation was continued. At appropriate times after labeling (30 to 120 min), samples were quickly poured into microcentrifuge tubes and centrifuged at room temperature for 20 s at 4,000  $\times$  g. The pellet was resuspended in 100  $\mu$ l of lysis buffer (20 mM Tris-HCl [pH 7.4], 10 mM Na<sub>2</sub>EDTA, 20% glycerol, 1% sodium dodecyl sulfate [SDS], 4 mM diethyl pyrocarbonate, and 0.01% bromophenol blue) and boiled for 4 min to achieve complete lysis. Cell lysates were subjected to electrophoresis in a 5%–8% tandem polyacrylamide gel (PAG) (1 mm thick) containing 7 M urea as described previously (18). The gels were dried and autoradiographed overnight on X-ray film (Hyperfilm; Amersham) at –70°C.

**Isolation and purification of acid-induced RNA molecules.** For the preparation of purified acid-induced RNA, cultures were grown and labeled in a manner similar to that described above, except that 1 mCi of H<sub>3</sub><sup>32</sup>PO<sub>4</sub> per ml of culture was added after the acid shift, and cells were incubated for an additional 2 h. Then, 2 volumes of the ice-cold stop solution (80% ethanol, 0.1% diethyl pyrocarbonate, 0.3 mM aurintricarboxylic acid) was added. Cultures were kept on ice for 20 min and harvested. The pellet was resuspended in 200  $\mu$ l of lysis buffer, boiled for 4 min, and electrophoresed as described above, in a 2-mm-thick gel. After electrophoresis the wet gel was exposed for 5 to 15 min to an X-ray film to detect the induced RNA bands by autoradiography. Appropriate portions of the gel were cut out with a razor blade. The gel slice was added on top of another 12% PAG. After electrophoresis, the bands were identified again by wet autoradiography and, if necessary, purified on the third gel (13% PAG). After two or three runs the RNA was eluted from the gel slice in 10 volumes of elution buffer (10 mM Tris-HCl, 10 mM NaCl [pH 7.2]) by vigorous shaking for 4 to 8 h at 4°C. The mixture was centrifuged for 30 min at 3,000  $\times$  g, the supernatant was applied to a PREPAC mini-column (Gibco BRL), and RNA was eluted according to the manufacturer's recommendations. RNA was precipitated with 1/10 volume of 3 M sodium acetate, pH 5.5, and 2.5 volumes of cold ethanol. The purity of RNA was verified on a 5%–8% PAG. We usually obtained 0.5  $\times$  10<sup>6</sup> to 1  $\times$  10<sup>6</sup> <sup>32</sup>P cpm of homogenic RNA from 5 to 7 ml of labeled culture.

**Hybridization with acid-induced RNA to the gene mapping membrane.** The gene mapping membrane (Takara Shuzo Co.) containing the recombinant lambda library from *E. coli* K-12 strain W3110 (29) was prehybridized for 18 h at 43°C in a solution containing 6 $\times$  SSC (1 $\times$  SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 5 $\times$  Denhardt's solution, 0.5% SDS, 50 mM phosphate buffer (pH 6.5), 50% formamide, denatured salmon sperm DNA (100  $\mu$ g/ml), and 0.1- $\mu$ g/ml concentrations each of 23S, 16S, and 5S rRNA. The <sup>32</sup>P-labeled RNA probe (0.5  $\times$  10<sup>6</sup> cpm) was added to the hybridization solution and hybridized for 18 h at 43°C. The membrane was washed at room temperature in 1 $\times$  SSC–0.1% SDS and then twice in 0.1 $\times$  SSC–0.1% SDS (30 min per wash) and exposed to X-ray film at –70°C overnight.

**Northern blot hybridization.** Total RNA from 30-ml cultures was isolated by the guanidine isothiocyanate method (52). Acid induction was carried out as described above. RNA (15 to 30  $\mu$ g) was electrophoresed in a 2% (wt/vol) formaldehyde-agarose gel. RNA was transferred to a nylon membrane (Hybond N; Amersham) according to the protocol described by Sambrook et al. (52). Hybridization to the labeled DNA probe and posthybridization washes were performed following the same protocol as described above.

**DNA methods.** Plasmid DNA was purified with a commercial plasmid purification kit (Qiagen) according to the manufacturer's recommendations. Small-scale plasmid preparations from *E. coli* were made by the method of Holmes and Quigley (25). Cloning procedures were performed generally according to standard protocols (52). *E. coli* cells were transformed as described by Nishimura et al. (43).  $\lambda$  phage DNA (clone 311) containing the *asr* gene from the Kohara library was prepared with the  $\lambda$ -magic DNA purification kit (Promega) according to the manufacturer's recommendations.

Restriction enzymes and other DNA- and RNA-modifying enzymes were from AB Fermentas, Gibco BRL, Boehringer Mannheim, and U.S. Biochemicals. All enzymes were used as recommended by the suppliers. Restricted DNA fragments were separated in 1.0 to 2.0% (wt/vol) agarose (U.S. Biochemicals) and were purified with Gene Clean (Bio 101, La Jolla, Calif.).

**Sequencing of the *asr* gene.** Plasmid pAS2 containing the *asr* gene was digested with *Pvu*II; the resulting 1.5-kb fragment was purified and PCR amplified with M13 forward (5'-GTTTTCCAGTACACGAC-3') and reverse (5'-AAACAGCTATGACCATG-3') sequencing primers (New England Biolabs). The resulting PCR products corresponding to the whole amplified *asr* DNA and to smaller fragments that had occurred by a fortuitous annealing of the primers to target DNA were sequenced as described by Krishnan et al. (30), with AmpliTaq polymerase (Perkin-Elmer Cetus). The M13 forward primer was found to anneal within a 1.5-kb noncoding *asr* DNA strand to a sequence complementary to the sequence 5'-GTTAACCCGATCAGAC-3' (upstream from the *asr* DNA sequence [not shown]). The M13 reverse sequencing primer was found to anneal to the sequences complementary to *asr* DNA sequences 5'-GAGGGTATGAC AATG-3' and 5'-AAGCATCATAAAAATA-3' (nucleotides [nt] 116 to 132 and 312–328 of the *asr* DNA sequence [see Fig. 3]). To allow the closure of a gap in the sequence near the left end of the *asr* DNA fragment and to determine the sequence of the second strand, synthetic custom oligonucleotide primers 5'-CTGGTGGTAATTATGATTA-3', 5'-AGGGGCTTTCTGTTCACC-3', 5'-TAGAATAACTGCGCATCA-3', and 5'-AACCCACTGCGGGGCCGT-3' (designed according to the previously determined sequence) were used.

The DNA sequence was analyzed for gene elements by using CDSB (54) and NNPP/Prokaryotic (49) software. Codon usage was evaluated by using codon adaptation index calculations (53). GenBank/EMBL, SwissProt, and PIR databases were searched for homologies by using the Gapped BLAST program (2). Prediction of protein signal sequence, cellular localization, and transmembrane regions was performed with the TopPredII (11) and PSORT software (41). Links to servers containing these programs are found at the Marseilles University ABIM W3 server reference page (37a).

**Allelic replacement of chromosomal *asr* gene.** The chromosomal knockout was performed essentially as described by Kulakauskas et al. (32). Plasmid pUC4K (Pharmacia) containing the *Tn903* Kan<sup>r</sup> gene on a 1.3-kb cassette was restricted by *Pst*I, the resulting fragment was ligated into *Pst*I-restricted plasmid pAS3 bearing the *asr* gene, and the reaction mixture was used to transform strain HB101. Positive clones were verified by electrophoresis of *Pst*I-digested plasmid DNA isolated from the transformants. The strain containing plasmid pAS4 with a mutagenized *asr* locus (*asr::Kan*) was infected with phage  $\lambda$  311 to generate the recombinant phage carrying a Kan insertion within *asr*. Phage  $\lambda$  311::Kan was used to infect strain N2212 to induce allelic replacement of the *asr* allele on the chromosome. The chromosomal *asr::Kan* mutants were verified by Southern hybridization using <sup>32</sup>P-labeled *asr* DNA fragment as a probe.

**PCR amplification.** PCR was carried out in a Perkin-Elmer Cetus thermocycler (model 480) with AmpliTaq polymerase (Perkin-Elmer Cetus). The cycling program was as follows: 30 cycles with a denaturation step at 94°C for 20 s, annealing at appropriate temperatures for 40 s, and elongation at 72°C for 1 min. PCR-amplified products were analyzed in 1 to 2% agarose gels.

**Plasmid insertional mutagenesis and *asr-lacZ* operon fusions with the mini-Mu bacteriophage transposon.** The procedures were performed as described by Groisman (20). Plasmid pAS6 containing the *asr* gene was used to transform strain POI1734 (9) harboring the mini-Mu element and a helper phage. The resulting strain containing pAS6 was used to prepare a lysate of mini-Mu. The lysate was used to transduce strain M8820 Mu cts (9). Transductants with an insertion in the *asr* gene on the plasmid were screened by plating the culture on an LPM that was buffered with 0.1 M MES (pH 5.0) and contained ampicillin (100  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml), and X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) (50  $\mu$ g/ml). After 36 h of incubation at 32°C, dark-blue colonies were picked up, and plasmid DNAs were isolated. Mu d insertions into the *asr* gene were verified by the electrophoresis of restricted plasmid DNA fragments and a subsequent hybridization to a <sup>32</sup>P-labeled DNA fragment with the *asr* gene. The exact location of insertions was determined by sequencing plasmid DNA with primers complementary to both ends of the inserted DNA fragment.

**Primer extension analysis.** Primer extension analysis was performed by using a 19-nt oligodeoxyribonucleotide (5'-ACAGACCCATAGCAGCGC-3') com-

plementary to the *asr* coding sequence 29 nt downstream of the mini-Mu insertion at position 127 in the *asr* DNA sequence given in Fig. 3. The procedures were generally performed as described by Sambrook et al. (52).

**Expression of *asr* and *phoB* genes.** To identify the *asr* gene product, the 706-bp *Csp6I* DNA fragment from plasmid pAS2 was subcloned into the expression vector pT7-6, yielding pAS5, and subsequently transformed into *E. coli* HB101 harboring plasmid pGP1-4 with a gene coding for T7 RNA polymerase (59). Strains containing pGP1-4 and recombinant plasmid or parental vector, pT7-6, as a control were induced and <sup>35</sup>S-labeled (Tran<sup>35</sup>S label; ICN Biomedical, Inc.) as described previously (59). Cell proteins (see below) were resolved on an SDS-PAGE (33), which was subsequently dried and exposed to X-ray film for 24 h.

For expression of PhoB protein in *E. coli* ANCH1 and ANCK10 cells, a 2.0-kb *Mlu*I fragment on plasmid pBC6 $\Delta$ *Pst*I (65) with the *phoB* gene was subcloned into the pT7-6 vector at the *Sma*I site downstream from the phage T7 polymerase promoter. PhoB expression from the resulting plasmid pVG6 was achieved by the temperature induction of T7 RNA polymerase (pGP1-4) as described by Tabor and Richardson (59).

**Preparation of cell extracts.** Cultures (300 ml) were grown in LB medium at 30°C to an optical density of 0.8 at 560 nm and harvested by low-speed centrifugation. Cell pellets were resuspended in sonication buffer (25 mM Tris-HCl [pH 7.6], 50 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, phenylmethylsulfonyl fluoride [100  $\mu$ g/ml]). Samples were sonicated with four 30-s bursts. Cell debris was removed by centrifugation at 12,000  $\times$  g, and the supernatant was used as a crude cell extract for mobility shift assays.

**Gel mobility shift assays.** The 181-bp *Csp6I-Pst*I DNA fragment containing the promoter region of the *E. coli asr* gene was <sup>32</sup>P labeled with a Klenow fragment. *E. coli* protein extracts were incubated with ~4,000 cpm of <sup>32</sup>P-end-labeled DNA fragment in the presence of 0.5  $\mu$ g of poly(dI-dC) · poly(dI-dC) and/or pBR322 DNA in a final volume of 20  $\mu$ l. Reaction mixtures were incubated at 37°C for 30 min in a solution of 10 mM Tris-HCl (pH 7.8), 50 mM KCl, 1 mM EDTA, bovine serum albumin (50  $\mu$ g/ml), 1 mM dithiothreitol, and 0.05% Nonidet P-40. After incubation, 2  $\mu$ l of loading buffer (50% glycerol in binding buffer plus 0.1 mg of bromophenol blue per ml) was added, and the samples were loaded immediately on a non-denaturing vertical 5% PAG with the current on. The electrophoresis buffer consisted of 10 mM Tris-HCl (pH 7.8)–1 mM EDTA. Following electrophoresis the wet gel was autoradiographed at –70°C overnight.

**Western immunoblot analysis.** *E. coli* proteins were separated by electrophoresis in an SDS–10% PAG, and fractionated proteins were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was incubated with rabbit polyclonal anti-PhoB antibody diluted 1:500. Binding of the primary antibody was evaluated by incubation with horseradish peroxidase-anti-rabbit immunoglobulin G conjugate (Amersham) and developing with 3,3'-diaminobenzidine tetrahydrochloride as described previously (3).

**Preparation of polyclonal anti-PhoB antibody.** *E. coli* ANCK10 harboring pVG6 and pGP1-4 was grown in LB medium to an  $A_{600}$  of 0.3. Cells were induced for PhoB synthesis by a temperature upshift, and incubation was continued for an additional 6 to 8 h. A crude cell extract was prepared by sonication as described above. Cell proteins were separated by SDS-PAGE electrophoresis (SDS-PAGE). The overexpressed PhoB protein was cut out after preparative gel electrophoresis, electroeluted, and used for rabbit immunization. The rabbits initially received a subcutaneous injection of 100  $\mu$ g of PhoB protein emulsified in Freund's complete adjuvant. Three booster injections of 40  $\mu$ g of purified PhoB protein emulsified in Freund's incomplete adjuvant were given once every subsequent 2 weeks. The rabbits were bled 2 weeks after the last injection, and the blood serum was analyzed for the presence of PhoB-specific antibody by Western immunoblot analysis. The nonspecific antibodies in the serum were adsorbed to a supernatant prepared from a sonicated culture of ANCH1 (*phoB*-*phoR*) strain according to the procedure of Yamada et al. (70).

**Alkaline phosphatase and  $\beta$ -galactosidase assays.** Alkaline phosphatase activity was determined as described by VanBogelen et al. (61), with *para*-nitrophenyl-phosphate as a substrate. Specific activity units are expressed as nanomoles of product formed per minute per cell culture optical density at 600 nm. For qualitative assay of alkaline phosphatase, agar plates were supplemented with XP (5-bromo-4-chloro-3-indolyl phosphate) (40  $\mu$ g/ml) as a chromogenic substrate.

$\beta$ -Galactosidase activity was determined from an *asr::lacZ* fusion strain according to the method of Miller (40).

**Nucleotide sequence accession number.** The nucleotide sequence for the *asr* gene has been submitted to GenBank and assigned accession no. L25410.

## RESULTS

**Identification of a unique RNA component inducible by low external pH.** An acid shift of growth media from pH 7.0 to a pH of 5.0 to 4.0 strongly induces a new RNA component, which could be identified on PAG after the electrophoresis of total in vivo <sup>32</sup>P-labeled *E. coli* RNA. Figure 1 shows the electrophoretic pattern obtained for small RNA molecules (50 to 1,000 nt) from extracts of cells grown in the LPM (pH 7.0)

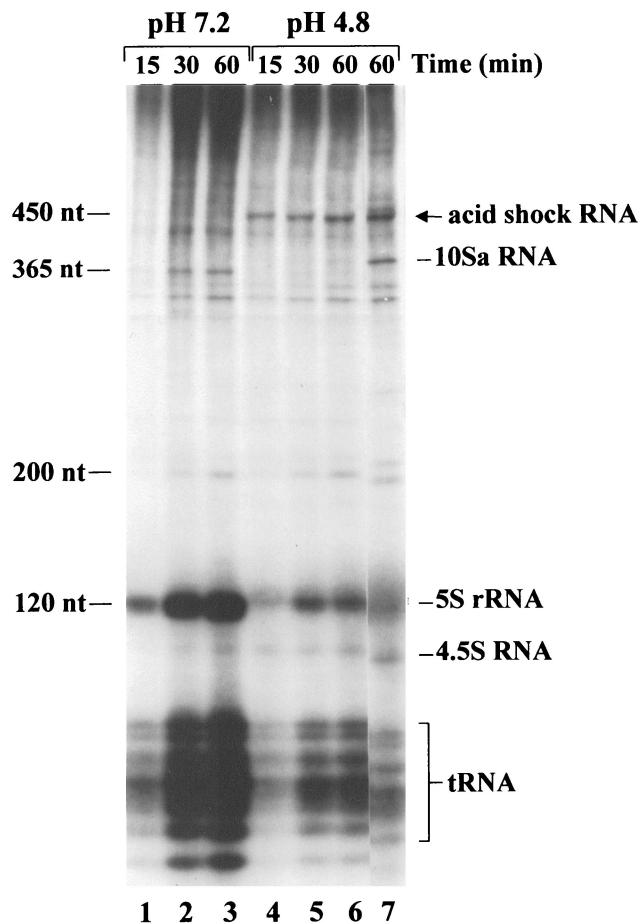


FIG. 1. Induction of acid shock RNA in *E. coli* K-12 strains. Cells were grown, acid induced, and  $^{32}\text{P}$  labeled as described in Materials and Methods. At the indicated times after the addition of  $^{32}\text{P}_i$  (added to the cultures at 30 min after shifting the pH of growth medium from 7.0 to 4.8, and added at the same time to control cultures [pH 7.0]), samples were taken and cell lysates were separated on 5%-8% polyacrylamide gel (the 5% part of the gel is not shown). Samples ( $2.5 \times 10^5$  cpm) were loaded on the gel. Lanes 1 to 3 and 4 to 6 contain RNAs derived from strain N2212 (10Sa RNA mutant) grown in LPM (pH 7.0) and after acid shift from pH 7.0 to 4.8, respectively. Lane 7 contains RNA derived from strain D10 (10Sa RNA-producing wild-type strain) grown in the same medium, pH shifted to 4.8. Various small *E. coli* RNA species are indicated. RNA lengths are shown at left. Acid shock RNA is indicated.

and after acidification to pH 4.8 (Fig. 1, lanes 1 to 3 and 4 to 7, respectively). The molecule, which we designated acid shock RNA, migrates in the region of two previously well characterized (10, 58) small *E. coli* RNAs, namely, 10Sa (365 nt [Fig. 1]) and its precursor, p10Sa ( $\sim 462$  nt [not shown]). According to the mobility on the gel, the approximate size of the newly identified RNA molecule lies between 400 and 450 nt. Acid-inducible RNA appears to be one of the most highly labeled small RNAs in the cell under the present conditions, indicating a high rate of synthesis. Accumulation of this RNA component upon acid shift was observed in all *E. coli* K-12 laboratory strains tested.

We analyzed the stability of the newly identified RNA in bacterial cells. *E. coli* D10 cells were grown in LPM, acid induced, and  $^{32}\text{P}$  labeled as described in Materials and Methods. Transcription initiation in the acid-induced *E. coli* culture (pH 4.8) was blocked by the addition of rifampin, and aliquots were removed at appropriate intervals for RNA preparation. We observed that acid shock RNA is long lived (Fig. 2) com-

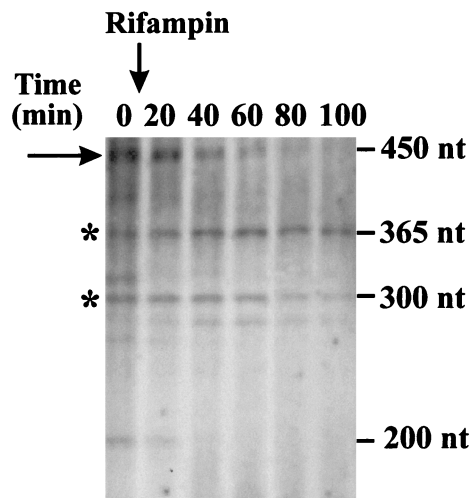


FIG. 2. Stability of acid shock RNA. Cells from strain D10 were grown in LPM (pH 7.0) to a density of  $\sim 10^8$  cells  $\text{ml}^{-1}$ , and then the pH was shifted down from 7.0 to 4.8 and the incubation was continued. Cells were  $^{32}\text{P}$  labeled as described in Materials and Methods. At 30 min after  $^{32}\text{P}_i$  addition, rifampin was added to the culture at a final concentration of 400  $\mu\text{g}/\text{ml}$ , and probes (2 ml) were sampled at appropriate intervals as indicated. Cell lysates ( $2.5 \times 10^5$  cpm) were separated on a 5%-8% polyacrylamide gel (only the 8% part is shown). Acid shock RNA is shown by the rightward-pointing arrow. Asterisks indicate the positions of stable *E. coli* 10Sa RNA (365 nt) and a stable unidentified RNA molecule ( $\sim 300$  nt). The downward-pointing arrow indicates the addition of rifampin to the culture. The lengths of *E. coli* RNAs are indicated on the right.

pared to other  $^{32}\text{P}$ -labeled bacterial transcripts and shows a half-life of  $>15$  min (stable *E. coli* 10Sa RNA and other unidentified stable *E. coli* RNA of  $\sim 300$  nt are indicated).

**Mapping and cloning of the *asr* gene.** In order to identify its coding locus on the *E. coli* chromosome, acid-induced RNA was purified as described in Materials and Methods. To avoid contamination with the 10Sa RNA molecule, which migrates in the gel close to the newly observed RNA, *E. coli* N2212 was chosen for purification. It contains a chromosomal insertion within the 10Sa RNA coding region, and the cells do not produce any detectable levels of this molecule (44). The purified acid shock RNA appeared as a single band when electrophoresed on a PAG (data not shown) and was used as a probe in hybridization to the lambda phage *E. coli* chromosomal library of Kohara et al. (29). One single phage clone ( $\lambda$  311) was found to hybridize to acid-induced RNA (data not shown). To further define the map position,  $^{32}\text{P}$ -labeled acid shock RNA was used as a probe to identify hybridizing bands among size-fractionated restriction digests of  $\lambda$  311 DNA. The smallest single fragment that hybridized to the RNA probe was an  $\sim 1.3$ -kb *PvuII*-*BglII* DNA fragment. The same result was obtained with *E. coli* chromosomal DNA digests (data not shown). These findings localized the acid shock RNA gene to 35.98 min on the *E. coli* chromosome between the *mlc* gene (35.9 min) and *pntBA* operon (36.06 min) (31). We designated the new gene *asr* (for acid shock RNA).

The 1.3-kb *asr* containing *PvuII*-*BglII* fragment from  $\lambda$  clone 311 DNA containing *asr* was subcloned into the pUC19 vector, yielding pAS2. *E. coli* N2212 bearing pAS2 was assayed for the ability to synthesize RNA upon acid shock. The amount of acid shock RNA upon acidification of the growth medium was only about twofold higher than that in the wild-type strain, as observed by in vivo  $^{32}\text{P}$ -labeled RNA PAGE (data not shown). The low expression level of *asr* when placed on the multicopy plasmid suggests a regulator-dependent manner of induction





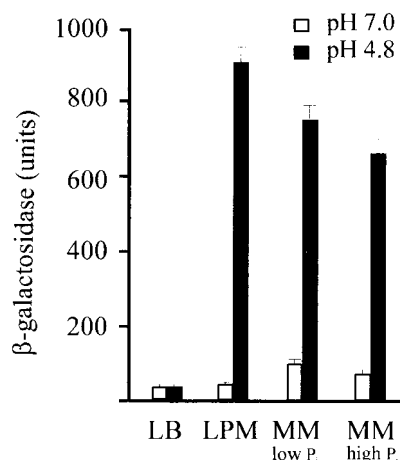


FIG. 6.  $\beta$ -Galactosidase activities from the strain containing the *asr::lacZ* fusion plasmid pAS8 in different growth media at pH 7.0 and upon acid shift from pH 7.0 to 4.8. The media used for analysis were LB medium, peptone-containing LPM, and MOPS MM with 0.1 or 1 mM phosphate (low and high  $P_i$ , respectively). Cells were grown and assayed as described in Materials and Methods. Values are given in Miller units. Each value corresponds to the mean of triplicate values (error bars, standard errors of the means).

not result in *asr* induction (not shown). These data suggest that *asr* is specifically induced by an external acidity.

Next, we examined the effect of growth medium on acid-induced expression of the *asr* gene. The peptone-containing LPM, which was used for in vivo  $^{32}P$  labeling (see Materials and Methods) and acid induction of the *asr* gene, contains less phosphate than rich LB broth and permits an efficient incorporation of the added  $^{32}P$  label (28). Starvation for phosphate as an additional stress stimulus might be involved in acid-induced expression of the *asr* gene. To examine a possible phosphate effect, duplicate sets of cultures of *E. coli* M8820 with *asr::lacZ* fusion in the plasmid pAS8 (Table 1) were grown in MOPS-buffered (pH 7.0) LB medium, in LPM, and in MOPS MM with either 0.01 or 1 mM phosphate (see Materials and Methods). After a shift from pH 7.0 to pH 4.8, *asr* expression was analyzed by measuring the  $\beta$ -galactosidase activity. The level of  $\beta$ -galactosidase was reduced approximately 10- to 15-fold in acid-induced cells grown in LB medium compared to those cultured either in LPM or in MOPS MM (Fig. 6). The results of  $\beta$ -galactosidase assay of acid-induced *E. coli* cells grown in MOPS MM with excess or limited phosphate show that phosphate limitation does not significantly influence the *asr* expression (Fig. 6). The phosphate stress conditions were confirmed by a >30-fold increase of alkaline phosphatase activity in *E. coli* cells grown in minimal medium (pH 4.8) with 0.01 M phosphate ( $108 \pm 4$  U [mean  $\pm$  standard error of the mean for three values]) compared to those grown in the same medium with 1 mM phosphate ( $3.2 \pm 0.06$  U).

**The *phoB-phoR* deletion mutant fails to induce acid shock RNA.** While testing the phosphate effect on *asr* expression, we also examined a possible involvement of *phoB-phoR*, a two-component regulatory system that regulates expression of a number of *E. coli* genes (the *pho* regulon) known to be inducible by phosphate starvation (64). The *phoR* product is a protein-histidine kinase, which when autophosphorylated (at phosphate starvation) phosphorylates PhoB, a transcriptional activator of genes belonging to the *pho* regulon (34, 35). The mutant strain ANCH1 with a chromosomal deletion of the entire *phoB-phoR* operon (70) was tested for the ability to induce acid shock RNA upon a shift from pH 7.0 to 4.8. We

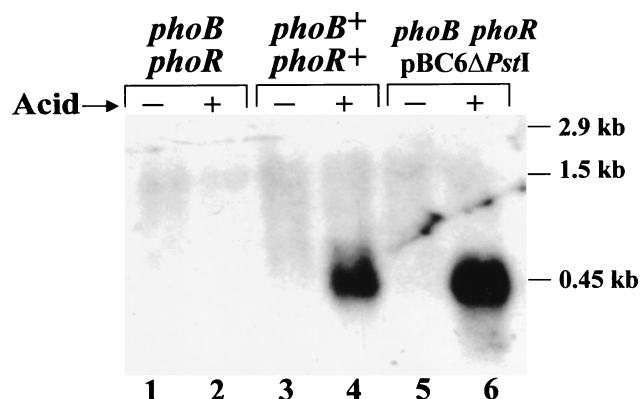


FIG. 7. Northern blot analysis of total cellular RNA isolated from strain ANCH1 ( $\Delta phoB-phoR$ ) (lanes 1 and 2), strain ANCK10 (wild type) (lanes 3 and 4), and strain ANCH1 with plasmid pBC6 $\Delta PstI$  (lanes 5 and 6). Cells were grown in the LPM, pH 7.0 (-), and after acid shift from pH 7.0 to 4.8 (+). RNA (30  $\mu$ g) was loaded into each lane. A  $^{32}P$ -labeled 0.7-kb *E. coli* DNA fragment containing the *asr* gene was used as a hybridization probe. Positions of the *asr* mRNA ( $\sim 0.45$  kb) and RNA markers (2.9 and 1.5 kb) are indicated.

found that the transcription of *asr* was completely abolished in the *phoB-phoR* deletion mutant in contrast to the wild-type strain ANCK10 (Fig. 7, lanes 2 and 4, respectively). The same results have been obtained with the ANCH1 strain containing either *phoB* (pVG12) or *phoR* (pVG11) (data not shown). Introduction of the plasmid pBC6 $\Delta PstI$  with cloned *E. coli phoB-phoR* operon into strain ANCH1 restored the acid-mediated induction of the *asr* gene (Fig. 7, lane 6).

The regulatory protein PhoB is known to bind specifically to a conserved DNA region consisting of 18 nt (the Pho box), which is a part of promoters for the genes belonging to the *pho* regulon (35). Upstream from the  $-10$  region of the *asr* promoter there is an 18-nt region (CTCACGGAAGTCTGCCAT [Fig. 3]), which is similar to the consensus sequence (CTGTCATAAACTGTCAT) of the Pho box. A 10-base space between the  $-10$  region and the Pho box is common (35). The  $-10$  sequence assigned for the *asr* promoter (TATAGT) is located 9 bases from the proposed Pho box (Fig. 3).

We analyzed whether the *asr* DNA fragment containing the proposed Pho box is capable of binding PhoB protein in vitro. The 181-bp *Csp61/PstI* DNA fragment with a promoter region of the *asr* gene was  $^{32}P$  labeled and analyzed for binding with *E. coli* proteins extracted from ANCH1 cells ( $\Delta phoB-phoR$ ) and from the same cells overexpressing PhoB protein (see Materials and Methods). Results of electrophoretic mobility shift assays are presented in Fig. 8. Incubation of the *asr* DNA fragment with protein extracts prepared from PhoB-producing *E. coli* ANCH1 cells resulted in the formation of two  $^{32}P$ -labeled *asr* DNA-protein complexes, C1 and C2 (Fig. 8A). The complex with the lower electrophoretic mobility, C1, was not observed with proteins of ANCH1 ( $\Delta phoB-phoR$ ) cells (Fig. 8A, lanes 5 to 7). Analogous binding results have been obtained with the  $^{32}P$ -labeled *E. coli phoB* promoter DNA fragment containing the Pho box, which was used as a positive control (36) (data not shown). The binding was effectively competed in the presence of an excess of unlabeled *asr* DNA, but not plasmid pBR322 DNA or poly(dI-dC)  $\cdot$  poly(dI-dC) (data not shown). No specific retarded complex was observed when a promoterless *asr* DNA fragment was used (data not shown).

Proteins present in the retarded  $^{32}P$ -labeled *asr* DNA-protein complexes C1 and C2 were examined by Western blot

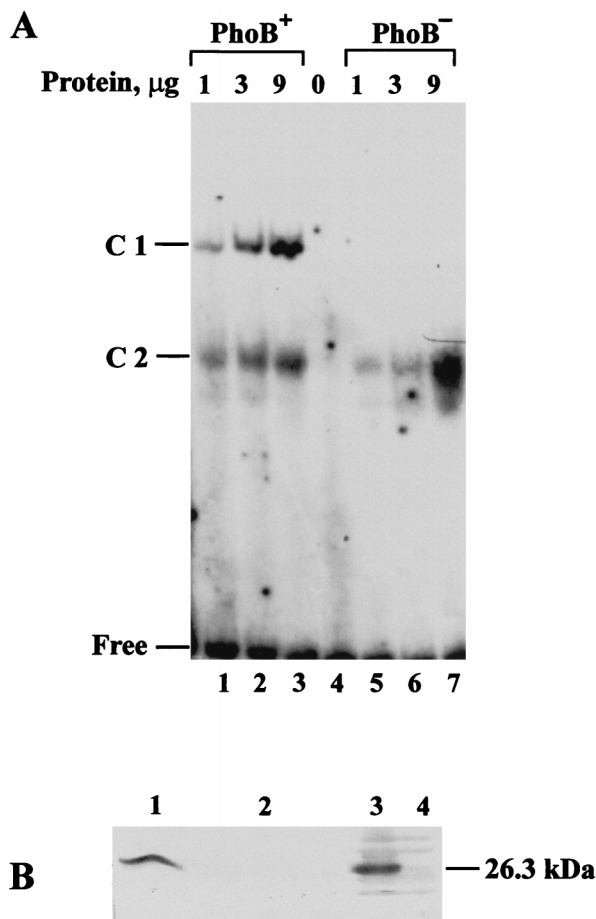


FIG. 8. (A) Gel mobility shift analysis for the binding of *E. coli* proteins to a promoter of the *asr* gene. Binding conditions are described in Materials and Methods. Increasing concentrations of the proteins from PhoB-producing ANCH1 cell extracts (PhoB<sup>+</sup>) (lanes 1 to 3) and the same cells with the expression vector pT7-6 only (PhoB<sup>-</sup>) (lanes 5 to 7) were incubated with <sup>32</sup>P-labeled *asr* promoter DNA. Reaction mixtures were loaded on a 5% nondenaturing PAG and electrophoresed. The gel was analyzed by autoradiography. Positions of the free <sup>32</sup>P-labeled *asr* DNA and DNA-protein complexes C1 and C2 are indicated on the left. (B) Western blot analysis of the proteins bound to the *asr* promoter DNA in complexes C1 and C2. Gel fragments with the retarded <sup>32</sup>P-labeled *asr* DNA C1 and C2 were cut from the PAG, denatured, and polymerized into SDS-10% PAG (lanes 1 and 2, respectively). As controls, protein extracts (5 μg) obtained from PhoB-producing ANCH1 cells (PhoB<sup>+</sup>) (lane 3) and the same cells with the expression vector pT7-6 only (PhoB<sup>-</sup>) (lane 4) were precasted in the similar fragments of the nondenaturing PAG, denatured, and polymerized side-by-side. After electrophoresis, separated proteins were analyzed with the polyclonal antibodies raised against PhoB protein. The position and molecular mass of the PhoB protein identified in the *E. coli* cell extract are indicated on the right.

analysis using polyclonal antibody raised against PhoB protein (see Materials and Methods). Anti-PhoB antibody specifically interacts with the protein released from the *asr* DNA-protein complex C1 (Fig. 8A, lanes 1 to 3). According to the electrophoretic mobility under SDS-PAGE conditions, the molecular mass of this polypeptide is identical to that of PhoB protein (26.3 kDa) as determined by Western blot analysis in PhoB-producing *E. coli* protein extracts (Fig. 8B, lane 3). These results indicate that *E. coli* activator protein PhoB is capable of binding *asr* promoter DNA in vitro, suggesting that this interaction is important for the *asr* transcription, which is also supported by the lack of *asr* transcript in *phoB* mutant cells. A faster migrating *asr* DNA-protein complex, C2, observed with

the *E. coli* proteins derived from PhoB-producing (ANCH1/pVG6) and *phoB* mutant (ANCH1) cells (Fig. 8A, lanes 1 to 3 and 5 to 7, respectively), most probably is formed by another *E. coli* protein, which is not recognized by the anti-PhoB antibody but shows some affinity to the target *asr* DNA sequence.

## DISCUSSION

Acid shift of growth medium from pH 7.0 to a pH of 5.0 to 4.0 strongly induces a novel *E. coli* RNA component which does not correspond to any previously characterized small RNA (27). Synthesis of the major small stable *E. coli* RNAs, such as 5S rRNA and tRNAs, is markedly decreased during acid shift as a consequence of a drop in growth and metabolic processes caused by acid stress. The relative amount of the acid shock RNA is comparable to that of pools of specific tRNAs and is about 1.5-fold higher than that of 10Sa RNA (10). The observed RNA under inducing conditions therefore represents one of the most abundant small RNA species in the cell. Rifampin decay experiments performed with acid-induced RNA demonstrate that it is long-lived bacterial RNA with a half-life of about 15 min.

The chromosomal location and organization of the *asr* region does not indicate *asr* to be a part of an operon known to be inducible by low external pH (38, 68) or presumably a part of any other *E. coli* operon (there are ~100- and 300-bp non-coding sequences extending from the respective sides of the *asr* locus). The *asr*-specific sequence has been observed in all tested *E. coli* laboratory K-12 strains as well as in enteropathogenic *E. coli* strains (data not shown).

The gene encoding acid shock RNA contains an ORF that, if translated, would yield an ~11.6-kDa polypeptide. Computer-assisted analysis demonstrated that the codons used in this ORF are highly preferred in *E. coli* protein-coding genes (53). Expression of the *asr* gene after transcription using the T7 RNA polymerase/promoter system defined a single polypeptide, which migrates in SDS-PAGE as an ~18 kDa protein. No synthesized protein has been observed upon induction of *E. coli* cells containing the expression vector only and cells with the truncated *asr* gene cloned in plasmid pT7-6 downstream from phage T7 RNA polymerase promoter. The molecular mass of the identified polypeptide does not exactly match the molecular mass of the polypeptide deduced from the *asr* DNA sequence. A reason for this disagreement might be physicochemical properties of the *asr* protein.

Furthermore, the first 30 amino acids of the deduced sequence possess all common features for a prokaryotic signal sequence, suggesting that the *asr* gene encodes a secretory protein. Hydrophathy profile analysis (43) (data not shown) of the putative polypeptide indicated the presence of one cluster of hydrophobic amino acids (amino acid positions 10 to 31 [Fig. 3]) located at its N terminus. According to the computer-predicted features, the *asr* polypeptide might be located either in the periplasm or in the outer membrane of the cell.

Analysis of the *asr* knockout mutant did not, however, shed light on a possible function for the *asr* gene product. Our data suggest that it is not essential for cell growth and survival under acid shock and its function is dispensable under the presently used growth conditions.

Most low-pH-inducible genes in *E. coli* and *S. typhimurium* were demonstrated to respond to other environmental stimuli (1, 13, 15, 55). Transcriptional analysis of the *asr* gene revealed that acid-mediated induction is significantly inhibited in complex (LB) medium compared to our LPM and MM, implying the existence of another regulatory component. Tests of individual constituents of LB medium indicated that yeast extract



inhibited pH-induced expression of the *asr* gene (data not shown), although its component responsible for the inhibition is currently unknown.

While starvation for phosphate as an additional stimulus was not found to influence expression of the *asr* gene, acid-triggered induction requires an intact *phoB-phoR* system controlling *E. coli* genes (*pho*) inducible by phosphate starvation (64, 67). Analysis of the *asr* gene revealed that its promoter region contains a sequence similar to the Pho box of PhoB-regulated promoters. DNA electrophoretic mobility shift experiments performed with protein extracts derived from PhoB-producing and *phoB* mutant strain demonstrated that PhoB protein indeed is able to bind a promoter DNA of the *asr* gene. All the above findings argue that *E. coli* might employ the two-component PhoB-PhoR regulatory system to mediate the low-pH-induced expression of the *asr* gene.

The intriguing question is this: how is acidity of the growth medium sensed? The periplasmic domain of the sensory kinase PhoQ of the *S. typhimurium* two-component regulatory system PhoP-PhoQ has been demonstrated to sense directly Mg<sup>2+</sup> cations in the periplasm (16, 17, 62). Recent observations in *Salmonella* argue that PhoQ is an acid shock protein and, possibly, senses pH (5). Some genes of the *E. coli* and *Bacillus subtilis* Pho regulons have been demonstrated to respond to other environmental factors, such as carbon, nitrogen starvation, anaerobiosis, UV light, and catabolites, although neither has the acidity of the medium as an inducer been reported nor has the involvement of a regulatory system *phoB-phoR* in transducing such environmental signals been reported (26, 66).

Treatment with weak acids able to permeate membranes, such as sodium benzoate and sodium salicylate, known to depress the cytoplasmic pH (51), did not result in *asr* induction (data not shown). Thus, the induction of *asr* occurs exclusively in response to external acidification, suggesting the existence of an external sensor.

We suggest a model in which H<sup>+</sup>, directly, or via its acceptor, might activate a sensor protein (PhoR) in the periplasm, which rapidly reflects changes in the extracellular pH. Signal is further transduced to an activator protein (PhoB) or to another regulatory factor, produced by the cell in response to acidification, or both. The interaction or cumulative action of PhoB protein and the putative factor lead to *asr* transcription. Such a model would explain the absence of the *asr* induction at neutral pH in the medium with low phosphate concentration, when the components of the *phoB-phoR* system are in abundance, as well as the presence of induction of the *asr* at low pH and high phosphate, when a low level of both proteins is present. Different models in which a yet-unknown factor regulates PhoR in response to acidification cannot be excluded, however. Further analysis will hopefully reveal new factors and their roles employed by bacteria facing acid stress.

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