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ArgR-Independent Induction and ArgR-Dependent Superinduction of the *astCADBE* Operon in *Escherichia coli*

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For *Escherichia coli*, growth in the absence of ammonia is termed nitrogen limited and results in the induction of genes that assimilate other nitrogen sources, a response mediated by σ^{54} and nitrogen regulator I (NR_I, also called NtrC). The *astCADBE* operon, which is required for growth with arginine as the sole nitrogen source, is moderately expressed during general nitrogen limitation and maximally expressed in the presence of arginine. The operon is also induced in stationary phase. Primer extension analysis of *E. coli* revealed the presence of a σ^{54}-dependent promoter utilized in exponential phase during nitrogen limitation and a σ^S-dependent promoter active during stationary phase. We used an *ast-lacZ* fusion to show that arginine stimulates expression, that ArgR, the arginine repressor, enhances expression from both promoters but is not essential, and that transcription by the two forms of the RNA polymerase is competitive and mutually exclusive. We demonstrated the binding of RNA polymerase holoenzymes, NR_I, and ArgR to the promoter region in vitro. We also reconstituted transcription from both promoters with purified components, which confirmed the accessory role of ArgR for the σ^{54}-dependent promoter. Thus, the *ast* operon exhibits nitrogen source-specific induction that is unique for an NR_I-dependent gene. The transcriptional regulation of the *ast* operon in *E. coli* differs from that in *Salmonella enterica* serovar Typhimurium, in which ArgR is required for *ast* operon expression.

Escherichia coli can utilize several compounds as the sole source of nitrogen in defined minimal media (33). Growth without ammonia, the compound that gives the fastest growth rate, induces proteins that transport and catabolize other nitrogen sources and assimilate the resulting nitrogen (25, 33). Alternative sigma factor σ^{54} and σ^{54}-dependent activator nitrogen regulator I (NR_I, also called NtrC) mediate this nitrogen-regulated (Ntr) response (25, 33).

Most Ntr genes that have been studied thus far are expressed in nitrogen-limiting (ammonia-lacking) media, regardless of the alternate nitrogen source present. Exceptions are some genes regulated by the σ^{70}-dependent activator, Nac, itself the product of an Ntr gene (4). Such nitrogen source-specific regulation of Nac-dependent genes can be the result of a specific regulator that responds to a particular nitrogen source (4; S. Ruback and L. Reitzer unpublished results). Such regulation is common for σ^{70}-dependent promoters but is unusual for σ^{54}-dependent promoters. An exception appears to be the *astCADBE* operon. The genes of this operon encode the enzymes of the arginine succinyltransferase (AST) pathway and are required for growth of *E. coli* with arginine as the nitrogen source (40). Gene array analysis showed that expression of the *ast* operon is NR_I dependent and Nac independent (52). Although arginine is not required for expression, arginine stimulates maximal expression (40). Expression of the *ast* operon in *Salmonella enterica* serovar Typhimurium implicated the arginine repressor protein, ArgR (23). ArgR is a homohexameric protein that, when complexed with arginine, represses arginine biosynthetic genes by binding at sites overlapping their promoters (24). Similar regulation of the *E. coli ast* operon could explain the arginine-specific induction.

In addition to Ntr control, the *ast* operon is induced by stationary phase, conditioned media, and carbon starvation (2, 5). Such regulation is physiologically advantageous since the resulting arginine catabolism produces citric acid cycle intermediates. Stationary-phase sigma factor σ^S has been implicated in this induction (15, 23).

We undertook a detailed in vivo and in vitro analysis of *astCADBE* expression and regulation. We verified the presence of two promoters, presented evidence that competition between these promoters exists, and obtained genetic and biochemical evidence for a nonessential accessory role of ArgR in the NR_I-dependent expression of the σ^{54}-dependent promoter. We also show that the regulation of the *ast* operon in *E. coli* differs significantly from that in *S. enterica* serovar Typhimurium.

(This work is a partial fulfillment of the requirement for a Ph.D. degree at the University of Texas at Dallas, Richardson, Tex., for A. Kiupakis.)

MATERIALS AND METHODS

Strains and plasmids. All strains used for assays in this study are derivatives of *E. coli* K-12 strain W3110 (41). Strains and plasmids used are listed in Table 1, and oligonucleotide primers are listed in Table 2.

The *ast* promoter was isolated from genomic DNA by PCR with primers XTH1 and SOT3 and cloned into the *Eco*RI site of pUC18, giving plasmid pUC-astp, and the sequence of the insert was verified. This insert was cloned into the *Eco*RI site of pTE103 to give plasmid pAK10, which was used for in vitro transcription. A 410-bp *Eco*RI/*Cla*I fragment from pUC-astp was blunted and cloned into the *Hin*cII site of pUC18 in both orientations to give plasmids pUC-astp(+) and pUC-astp(−). *Eco*RI/*Pst*I fragments from these plasmids were used for DNase I footprinting.

An *ast-lacZ* fusion was constructed by cloning the insert of pUC-astp into the

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype or description	Source or reference
Strains		
W3110 (wild type)	*lacL8 lacI*^{q}	Laboratory strain
AK15	W3110 *trp*::*putPA1303*::[Kan^r-*astC*′-′*lac*]	This study
AK16	W3110 *trp*::*putPA1303*::[Kan^r-*astC*′-′*lac*] *rpoS*::*tet*	This study
AK17	W3110 *trp*::*putPA1303*::[Kan^r-*astC*′-′*lac*] *himD*::*cam*	This study
AK18	W3110 *trp*::*putPA1303*::[Kan^r-*astC*′-′*lac*] *argR*::*cam*	This study
AK21	W3110 *trp*::*putPA1303*::[Kan^r-*astC*′-′*lac*] Δ*crp* Str^r	This study
AK22	W3110 *rpoN*::FRT-Kan^r-FRT	This study
AK23	W3110 Δ*rpoN*	This study
AK24	W3110 *trp*::*putPA1303*::[Kan^r-*astC*′-′*lac*] Δ*rpoN*	This study
AK26	W3110 *trp*::*putPA1303*::[Kan^r-*astC*′-′*lac*] Δ*rpoN rpoS*::*tet*	This study
CA8445	Δ*crp* Str^r	37
K5746	Overproduces both IHF subunits	30
TE2680	Δ(*lac*)*X74 recD1903*::Tn*10 trpDC700*::*putPA1303*::[Kan^s-Cam^r-′*lac*]	13
K4633	*recD*::*tet*	D. Friedman (University of Michigan)
BW12848	*himD*::*cam*	B. Wanner (Purdue University)
UM315	*rpoS*::*tet*	Laboratory strain originally obtained from P. C. Loewen
Plasmids		
pRS551	Amp^r Kan^r-′*lac*	42
pTE103	Amp^r	14
pAK10	Amp^r *astC*p	This study
pUTmini-Tn*5* Cm	Amp^r MCS-*cam*-MCS	10
pQErpoS30	Amp^r p*tac*::His6-*rpoS*	3
pTH7	Amp^r p*tac*::*rpoN*	19
pXZCRP	Amp^r *crp*	51

*Eco*RI site of pRS551. An 8.5-kb *Xho*I/*Sac*II fragment of the resulting plasmid was transformed into strain TE2680 to place the *ast-lacZ* fusion into the *trp* operon of *E. coli*. The fusion was moved into W3110 by P1 transduction of kanamycin resistance to give strain AK15.

We constructed an *rpoN* derivative of W3110 by P1 transduction of the *rpoN208*::Tn *10* allele from strain YMC18 (46), but the resulting strain did not exhibit the correct phenotype, e.g., it grew with alanine or arginine as the sole nitrogen source (this has been observed before [15]). We decided, therefore, to construct an in-frame deletion of the *rpoN* gene using the method of Datsenko and Wanner (9). We transformed a PCR product obtained from plasmid pKD13 with primers rpoN(f)-P4 and rpoN(r)-P1 into W3110/pKD46. We removed the Kan^r cassette from the resulting insertion-deletion strain (AK22) by FLP recombination to give strain AK23. We isolated the *rpoN* allele of AK23 by PCR amplification with primers rpoN(f)1 and rpoN(r)1. We cloned the resulting fragment and sequenced the *rpoN* region to verify that the in-frame deletion removed the entire *rpoN* open reading frame (ORF) except of the first two codons and the stop codon. The resulting 30-amino-acid product of the ORF shows no homology to anything in the databases. The *ast-lacZ* fusion from AK15 was moved into AK23 by P1 transduction to give strain AK24.

TABLE 2. Oligonucleotide primers

Primer	Sequence
XTH1	GGCGA ATTCG GTGAG GTCTG GCGCG CAGGC CG
SOT3	GGCGA ATTCT CATCC ATTCA TCAAA GTTTT CACGC G
SOT4	GGATC CTGAG ACATA GCGAC CTCTA C
argR5	CCAGG ATCCG GAGTC GCATC TTCAC C
argR6	CCAGA ATTCT ATGCA AACAG TCAGC CC
argR7	GCTCA AGCTT AATCT CTGCC CCGTC G
argR8	CCACG GTACC AGCAT TTCAC GCATA TCC
himD1	GTAAT TCTCT GACTC TTCGG
himD2	CCCGA GGCAT ATTCA GAAG
CRP1	CAGAG GATAA CCGCG C
CRP2	ACGCG CCACT CCGAC G
RPOS1	CCGTA AACCC GCTGC G
RPOS2	TTGCG TGGTA TCTTC CGG
rpoN(f)-P4	TGCGA CGTTT TAGCA GGAGA GTACG ATTCT GAACA TGAAG ATTCC GGGGA TCCGT CGACC
rpoN(r)-P1	TGTTG AGCTG CATAG TGTCT TCCTT ATCGG TTGGG TCAAA GTGTA GGCTG GAGCT GCTTC G
rpoN(f)1	AGCGT CTATA CCTTG GGG
rpoN(r)1	TCTCG ACGTT ATTTC CGG

To disrupt *argR*, two genomic DNA PCR products, obtained with primer pairs argR5 and argR6 and argR7 and argR8, and a 3.6-kb *Eco*RI fragment from pUTmini-Tn5 Cm carrying the Cam^r gene were cloned consecutively into pBlue-Script (Stratagene) between the *Bam*HI and *Eco*RI sites, between the *Hin*dIII and *Kpn*I sites, and into the *Eco*RI site, respectively. The resulting *argR*::*cam* allele was recombined onto the chromosome by transformation of a 4.65-kb *Sac*I-*Kpn*I fragment into strain K4633 and then moved by P1 transduction into AK15 to give strain AK18. The disruption, which removes the entire *argR* ORF, was verified by genomic PCR using primers argR5 and argR8.

The *rpoS*, *himD*, and *crp* deletions were moved into AK15 by P1 transduction from strains UM315, BW12848, and CA8445, respectively, to give strains AK16, AK17, and AK20. The *rpoS*::*tet* allele of UM315 was also moved into AK24 to give AK26. The disruptions in these strains were verified by genomic PCR with primer pairs RPOS1 and RPOS2, himD1 and himD2, and CRP1 and CRP2.

Cell growth. Minimal media contained W salts (36), 0.02% thiamine, 0.4% (wt/vol) glucose or glycerol, and a nitrogen source (0.2%) (glutamine-arginine media contained 0.2% glutamine and 0.1% arginine). Overnight cultures (2 or 3 ml in various media) with the appropriate antibiotic were harvested, washed with 1 ml of 150 mM NaCl, resuspended in 0.5 ml of 150 mM NaCl, and used to inoculate cultures without antibiotics for assays. Unless otherwise specified, antibiotic concentrations were 100 μg/ml for ampicillin, 25 μg/ml for kanamycin, 15 μg/ml for chloramphenicol, 30 μg/ml for streptomycin, and 20 μg/ml for tetracycline. All cultures for assays were grown at 30°C.

RNA extraction. Ten-milliliter cultures were grown to a density of ~80 Klett units (filter 42) for exponential-phase assays or grown to stationary phase and harvested the next morning. The flask was moved to −80°C until the culture became slushy, and the cells were harvested by centrifugation. The cell pellets were used immediately or frozen at −80°C for no longer than 2 days. RNA was extracted as described previously (1) with the following modification: after addition of the acidic phenol (pH 4.2; Sigma) the solution was incubated at 60°C for

10 min with vortexing every 2.5 min. The RNA was quantitated by measuring A_{280}.

Primer extension analysis. Primer extension experiments were carried out essentially as described previously (38), except that 10 or 15 μg of total RNA was dried in a SpeedVac and resuspended in 5 to 10 μl of the ^{32}P-labeled primer (17 to 34 pmol) and the solution was evaporated in a SpeedVac until just dry before resuspension in the hybridization solution. The reaction mixture did not contain actinomycin. After the reaction 10 μl of sequencing loading dye (80% formamide, 1 mM EDTA [pH 8.0], 0.2% [wt/vol] xylene cyanol, 0.2% [wt/vol] bromophenol blue) was added to the reaction tubes, which were placed on ice until they were heated and the contents were loaded on a sequencing gel.

β-Galactosidase assays. Cells were grown in the various media containing 0.01% tryptophan. For exponential-phase measurements, 10-ml cultures were grown to a density of 90 to 120 Klett units. Stationary-phase measurements were from 2-ml cultures grown for 36 to 48 h. Cells were harvested by centrifugation and washed with 1 ml of 150 mM NaCl, and cell pellets were frozen for no longer than 2 days. Cells were thawed on ice, resuspended in 0.5 ml of 50 mM $NaPO_4$, pH 7.5–1 mM β-mercaptoethanol, and lysed by sonication. After centrifugation at 4°C, the supernatants were used for β-galactosidase assays as described previously (28). The protein concentrations of the extracts were determined with bovine serum albumin as a standard (22).

Proteins. For purification of σ^{54}, W3110 carrying plasmid pTH7 was grown to late exponential phase in 6 liters of Luria broth (LB) with 0.5 mg of ampicillin/ml, incubated with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h, harvested by centrifugation, and washed with 500 ml of 150 mM NaCl, and the cell pellet was stored at −80°C. All subsequent steps were done at 4°C. Cells were thawed, resuspended in 60 ml of buffer A (20 mM Tris-HCl [pH 7.5], 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], 10% glycerol) with 1 mM phenylmethylsulfonyl fluoride and lysed by sonication. Streptomycin sulfate was added to the clarified lysate to 30 mg/ml, the lysate was stirred for 30 min, and the insoluble material was removed by centrifugation. The 40 to 65% $(NH_4)_2SO_4$ precipitate was resuspended in 3 ml and dialyzed twice against 1 liter of buffer A, loaded on a Mono-Q column (Pharmacia), and eluted with a 100 to 550 mM KCl gradient. RpoN fractions (0.30 to 0.45 mM KCl) were pooled, precipitated with 70% ammonium sulfate, resuspended in 0.2 ml of buffer B (20 mM $NaPO_4$ [pH 7.0], 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol), dialyzed against the same buffer, concentrated on a Centricon (Amicon), and loaded on a Sephadex column (Pharmacia). Fractions containing σ^{54} were again pooled and precipitated, resuspended in buffer C (20 mM $NaPO_4$ [pH 7.0], 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 1 M $(NH_4)_2SO_4$, and loaded on a phenyl-Sepharose column (Pharmacia). RpoN eluted close to the end of a 1 to 0 M $(NH_4)_2SO_4$ gradient. The protein was precipitated with ammonium sulfate, resuspended, dialyzed in buffer A, concentrated, and stored at −80°C with 40% glycerol. The final preparation appeared to be over 90% pure by visual inspection after gel electrophoresis.

RpoS was purified as described previously (3) from W3110 carrying plasmid pQErpoS30 except that the cells were lysed by sonication, RpoS was precipitated from the clarified cell lysate with 40% $(NH_4)_2SO_4$, chromatography was done in batch in buffers containing 600 mM KCl, and the binding buffer contained 10 mM imidazole.

The cyclic AMP (cAMP) receptor protein (CRP) was purified by modifications of previously described procedures (6, 12, 17). A 1-liter culture of strain CA8445/pXZCRP was grown in LB with ampicillin at 37°C overnight, harvested, washed with 0.5 liter of 150 mM NaCl, and resuspended in 30 ml of buffer D (20 mM KPO_4 [pH 7.7], 40 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol) with 10 mM phenylmethylsulfonyl fluoride. After sonication, 0.5% Polymin P was added to the clarified supernatant and the mixture was stirred for 10 min and then centrifuged. The 42 to 60% $(NH_4)_2SO_4$ pellet of the Polymin P supernatant was resuspended in buffer D, pH 6.7, dialyzed overnight against 1 liter of the same buffer, and loaded on a 7-ml BIO-REX 70 column (Bio-Rad), and CRP was eluted with a 100 to 700 mM KCl gradient. CRP-containing fractions were pooled, concentrated, and dialyzed twice against 1 liter of buffer TG (50 mM Tris-HCl [pH 8.15], 1 mM EDTA, 1 mM DTT, 10% glycerol). The sample was loaded by gravity on a 1.5-ml DEAE-Spherilose column (ISCO), and CRP was collected in the flowthrough, concentrated, and stored at −80°C. When we used the purified CRP for in vitro transcription, we discovered that it contained an RNase activity. To remove this activity, our CRP preparation was subjected to gel filtration through a Sephadex column (Pharmacia), which resulted in the separation of two RNase activities, one of which coeluted with CRP. Fractions containing CRP were pooled, concentrated by ammonium sulfate precipitation, resuspended in buffer E (20 mM Tris-HCl [pH 8.8], 150 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol), dialyzed against the same buffer, and loaded on a Mono-Q column (Pharmacia), and CRP was collected in the flowthrough. The final preparation was >90% pure, did not contain any detectable RNase activity, and was active in a mobility shift assay using a 40-bp oligonucleotide containing the CRP consensus binding sequence.

NR_I (29) and integration host factor (IHF) (30) were purified as described previously, and ArgR was a generous gift from Greg VanDuyne.

DNase I footprinting. DNase I footprinting assays were performed as described previously (7). Footprinting with ArgR was done in the presence of 5 mM arginine.

Mobility shift assays. These assays were done in 25 μl containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 5 mM arginine, 100 μg of acetylated bovine serum albumin/ml, 0.6 μg of sonicated salmon sperm DNA/ml, 10% glycerol, 0.1 nM ^{32}P-labeled oligonucleotide, and various concentrations of the ArgR protein. The tubes were incubated for 20 min at room temperature and then loaded immediately on a 4% (49:1) polyacrylamide gel containing 40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 5 mM arginine in both the gel and the running buffer. The gel was run at 8 V/cm for 1.5 h, followed by 4 V/cm for 3.5 h.

In vitro transcription. Single-round in vitro transcription reaction mixtures contained, in a final volume of 25 μl, transcription buffer (50 mM Tris-HCl [pH 7.5], 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA), 5 mM arginine (when indicated in Fig. 10A and in all lanes in Fig. 10B), 5 mM cAMP (for Fig. 10B), 10 nM pAK10 as a template, 4 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.1 mM UTP, 1.25 μl of [α-^{32}P]UTP (800 Ci/mmol, 10 mCi/ml; ICN), 150 μg of heparin, 100 nM core RNA polymerase (Epicenter Technologies), 300 nM σ^{54} or σ^S, 50 nM NR_I dimer, 50 nM NR_{II} dimer, 50 nM ArgR hexamer, 100 nM IHF, and various concentrations of CRP.

A mixture containing core polymerase, sigma factor, arginine, cAMP and 3 U of RNAGuard (Pharmacia) in transcription buffer was incubated for 5 min at 37°C. NR_I, NR_{II}, and ATP were added, and the mixture was incubated for another 10 min. The mixture was incubated for 10 more minutes after addition of ArgR and IHF in that order for reactions with σ^{54} or of ArgR and CRP simultaneously for reactions with σ^S. All components were in transcription buffer, and the final volume after these additions was 20.5 μl. Transcription was initiated by addition of 4.5 μl of a solution containing GTP, CTP, cold and hot UTP, and heparin in transcription buffer and was allowed to proceed for 15 min at 37°C, after which the reactions were stopped by addition of 25 μl of 50 mM EDTA-100 μg of bakers' yeast tRNA/ml and the reaction mixtures were stored on ice. The reaction mixtures were extracted with 50 μl of acidic phenol (pH 4.2; Sigma), and 40 μl of the upper phase was added to 10 μl of sequencing loading dye. The tubes were heated to 90°C for 3 min and centrifuged briefly before the contents were loaded on a 5% acrylamide–8 M urea–0.5× Tris-borate-EDTA gel.

RESULTS

Expression of the *ast* genes is regulated at two promoters. Direct assays of the AST pathway enzymes have shown that general nitrogen limitation, the specific nitrogen source, and the carbon source affect *ast* operon expression (40). Additionally, other reports published during the course of this work found that the growth phase also affects expression of the operon in *E. coli* (2, 5) and *S. enterica* serovar Typhimurium (23) and that σ^{54} and σ^S are both involved in this regulation. To analyze in more detail the regulation of the *ast* operon, we performed primer extension analysis on *E. coli* total RNA isolated from cells grown in different media and harvested at different phases of growth. As shown in Fig. 1, exponentially growing cells in glucose-arginine minimal medium expressed the *ast* operon from an upstream promoter (which will be shown to require σ^{54}), while stationary-phase cells in complex media (LB) expressed the operon from a second promoter (which will be shown to require σ^S). Transcription from the σ^{54}-dependent promoter initiated at two adenines found 2 bp apart in the promoter sequence. It is possible that the shorter primer extension product is a degradation product of the longer one. The transcriptional start site for the σ^S-dependent

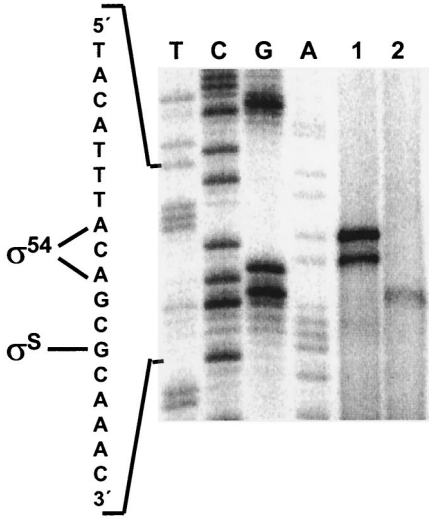

FIG. 1. Start site mapping of the *ast* promoter. Extension of primer SOT3 was performed with RNA isolated from W3110 cells growing exponentially on glucose-arginine minimal medium (lane 1) or in stationary phase after growth in LB (lane 2), and the reaction products were run next to a sequencing ladder. The promoter sequence and the start sites for the σ^{54} and σ^{S} promoters are shown.

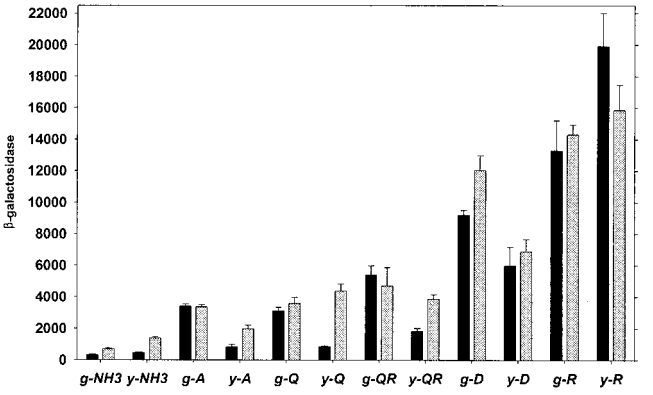

FIG. 3. In vivo expression of the *ast* operon. Strain AK15 was grown on a variety of minimal media, and β-galactosidase levels in exponential (black bars) and stationary (gray bars) phases were measured. Units are nanomoles of product per minute per milligram of total soluble protein. Averages of at least three independent determinations and standard errors of the means are shown. Carbon sources: g, glucose; y, glycerol. Nitrogen sources are indicated with the one-letter amino acid designation.

promoter was a guanine 5 bp downstream from the most upstream σ^{54}-dependent start site.

The proximity of the initiation sites raised the question of competition between the two holoenzymes for binding to the *ast* promoters. To investigate this issue, we examined promoter utilization in cells grown on a more diverse collection of media, including different carbon and nitrogen sources, and in cells in both phases of growth. Cells growing exponentially in nitrogen-limited minimal media, especially those with arginine as the sole nitrogen source, utilized the σ^{54}-dependent promoter (Fig. 2). In contrast, stationary-phase cells grown in media with arginine but without glucose (glycerol-arginine minimal medium or LB) utilized the σ^{S}-dependent promoter. We never observed expression from both promoters simultaneously, and the case of glycerol-arginine medium is extreme--promoter utilization switched from the σ^{54}-dependent promoter during exponential phase to the σ^{S}-dependent promoter in stationary phase. This suggests that expression from the two promoters is mutually exclusive and that the two forms of the polymerase compete for binding.

In vivo analysis of *astCADBE* expression. To quantitatively analyze transcriptional regulation of the *ast* operon, we constructed a strain with an *ast* promoter-*lacZ* fusion in the *E. coli* chromosome. The fusion did not disrupt the wild-type *ast* operon, which permits growth with arginine as a nitrogen source. We assayed β-galactosidase activity in cells grown in minimal media with different carbon and nitrogen sources and harvested during exponential or stationary phase. We found that the *ast* operon was highly inducible, with a 60-fold range of expression (Fig. 3). This could not be attributed to differences in the growth rate, which showed only a sixfold range for the different media (not shown). As expected, nitrogen limitation increased expression, and there appeared to be specific induction, since β-galactosidase levels were not the same for all growth-limiting nitrogen sources but were highest when arginine was the sole source of nitrogen, followed by aspartate. These results agree with direct enzyme assays (40), which validates use of the *lacZ* fusion for expression studies. In cells

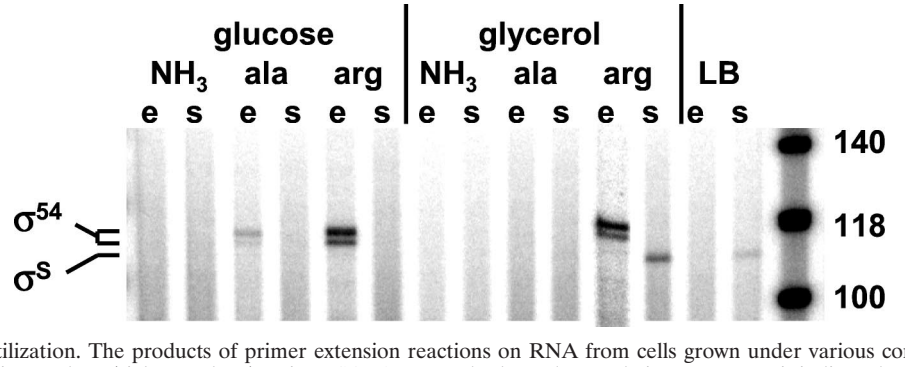

FIG. 2. Promoter utilization. The products of primer extension reactions on RNA from cells grown under various conditions were run on a sequencing gel next to size markers (rightmost lane). Primer SOT4 was used. The carbon and nitrogen source is indicated, as is the phase of growth (e, exponential; s, stationary). The promoter from which the transcripts originate is noted. (The glycerol-arginine exponential-phase lane is from a different gel and is inserted here for presentation purposes).

growing exponentially (Fig. 3) with a poor nitrogen source other than arginine, expression was higher with glucose as the carbon source than it was with glycerol, while the same was not always true in stationary phase (Fig. 3). Since expression under these conditions was σ^{54} dependent (Fig. 2), this observation suggests that transcription by σ^{54} is reduced in carbon-limited media. In general, activity was the same in exponentially growing and stationary-phase cells, although, as indicated above, this does not imply utilization of one promoter.

Factors affecting expression of the *ast* operon. Based on the expression analysis presented here a number of proteins could be involved in the transcriptional regulation of the *astCADBE* operon: the alternative sigma factors σ^{54} and σ^{S}, the σ^{54}-dependent activator NR_I, and CRP. Additionally, the arginine repressor, ArgR, has been shown to be involved in the transcriptional regulation of the *ast* operon in *S. enterica* serovar Typhimurium (23), and IHF is involved in the regulation of some NR_I- and σ^{54}-dependent promoters. To investigate the possible effect of each of these factors on the expression of the *ast* operon in *E. coli*, we constructed *rpoN*, *rpoS*, *crp*, *argR*, and *himD* (encoding one of the IHF subunits) mutants and an *rpoN rpoS* double mutant in a W3110 background and assayed the expression of β-galactosidase from cells with the *ast-lacZ* fusion in a variety of media. (The *rpoN* mutant is essentially a glutamine auxotroph in nitrogen-limited media and, therefore, could not be grown in many of the media used.)

(i) Loss of the sigma factors. The *rpoN rpoS* double mutant had basal levels of transcription for cells in all media, which implies that only σ^{54} and σ^{S} participate in the expression of the *ast* operon in *E. coli* (Fig. 4).

The *rpoN* mutant (σ^{54} deficient) had lower β-galactosidase activity than a wild-type strain, but only during exponential growth in nitrogen-limited media (glucose as the carbon source and either glutamine or glutamine plus arginine as the nitrogen sources) (Fig. 4). With glycerol as the carbon source, this mutant had essentially the same β-galactosidase activity as the wild-type strain (less than 20% difference for all media), which implies that this activity requires σ^{S} (Fig. 4). The *rpoN* mutant had higher activity than the wild type in stationary phase with ammonia and either glucose or glycerol. This is consistent with the hypothesis of holoenzyme competition (Fig. 4). Interestingly, the *rpoN* and *rpoN rpoS* strains do not show the reduced expression in glycerol-containing media described previously (Fig. 3 and 4). This may suggest that σ^{54} mediates the glycerol effect.

Loss of σ^{S} was most noticeable for cells grown in nitrogen-rich (ammonia-containing) media, regardless of the carbon source or growth phase (Fig. 4). Such growth results in minimal phosphorylation of NR_I, which should prevent activation of the σ^{54}-dependent promoter. For exponentially growing cells with glutamine, glutamine with arginine, alanine, aspartate, or arginine as the nitrogen source, deletion of *rpoS* had little effect on β-galactosidase activity (Fig. 4 and 5). These results were expected since NR_I is phosphorylated, which should activate the σ^{54}-dependent promoter. β-Galactosidase activities for the wild-type and *rpoS* strains harvested in stationary phase were similar (Fig. 5). This was unexpected since expression in W3110 should be primarily from the σ^{S}-dependent promoter (Fig. 2, glycerol-arginine medium). This may imply a switch in promoter utilization, from the σ^{S}-dependent promoter in the wild type to the σ^{54}-dependent promoter in the *rpoS* mutant. However, the accumulation of β-galactosidase during exponential growth may account for most of the activity, and there may be little or no *ast* operon expression during stationary phase in the *rpoS* mutant.

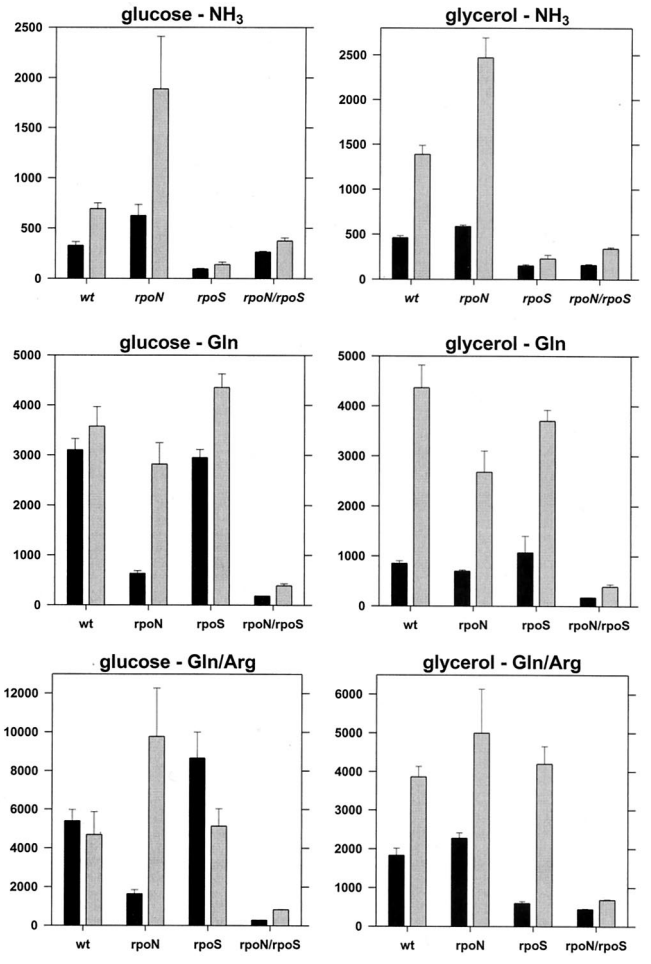

FIG. 4. Sigma factors and *ast* expression. Strains carrying the *ast-lacZ* fusion were grown on the indicated media and assayed for β-galactosidase activity in exponential (black bars) and stationary (gray bars) phases. Values are reported as in Fig. 3. Note the differences in the scaling of the *y* axes. wt, wild type.

(ii) ArgR. The *argR* deletion had a significant effect on transcription only under conditions that resulted in the highest levels of expression, i.e., with arginine as the sole nitrogen source (Fig. 5). Loss of ArgR appeared to prevent arginine-specific induction, which suggests that ArgR mediates this induction. Effectively, the *argR* deletion acted to limit the highest attainable level of expression to ~6,000 nmol min^{-1} mg of $protein^{-1}$. This was true for the exponential and stationary phases and suggests that, while not essential, ArgR enhances transcription at least from the σ^{54}-dependent promoters and from the σ^{S}-dependent promoter to the extent that β-galactosidase activity reflects synthesis in stationary phase and not from previous accumulation during exponential growth.

(iii) CRP. Deletion of *crp* lowered β-galactosidase activity, especially in stationary phase and in media in which there were

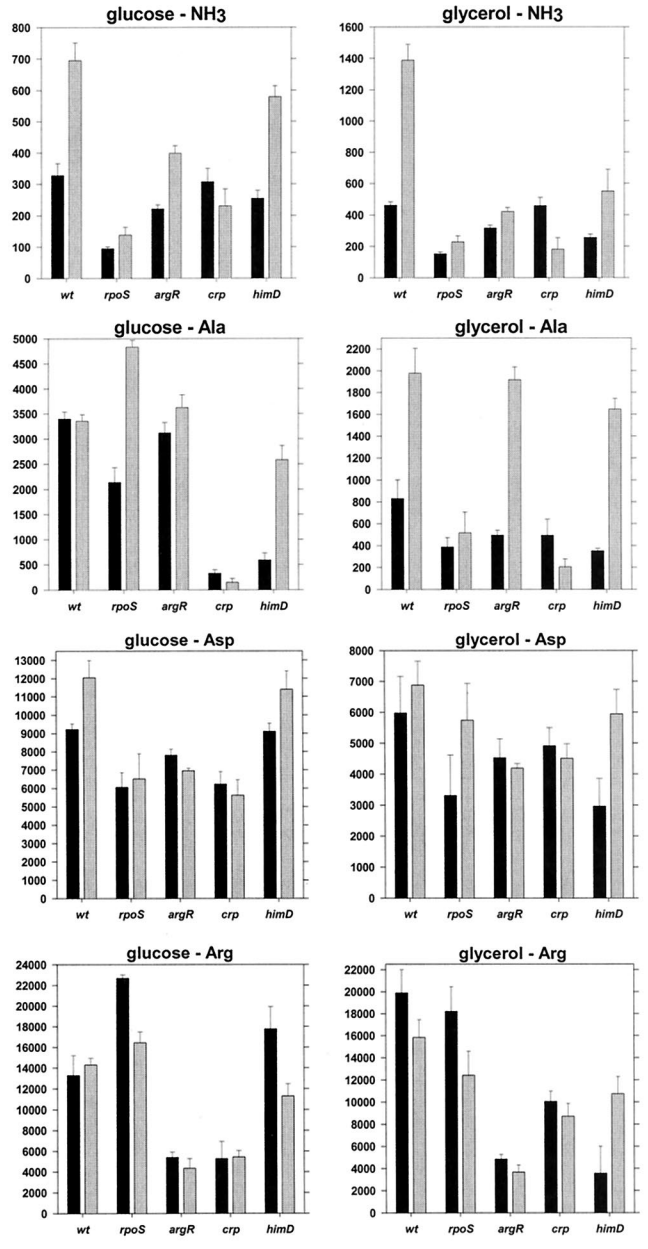

FIG. 5. Effects of regulatory mutants on *ast* expression. The presentation of the data is the same as that for Fig. 4. wt, wild type.

low levels of expression (ammonia or alanine as the nitrogen source) (Fig. 5). The *crp* mutant, like the *argR* mutant, seemed to limit the highest level of expression that could be achieved. However, this may have been an indirect effect of the slower growth of this mutant in most media.

(iv) IHF. Finally, the *himD* mutation did not have an appreciable effect on the β-galactosidase levels under most conditions (Fig. 5). Maximal activity was attainable (in glucose-arginine medium), and there was no discernible pattern in the cases where this mutation apparently impaired expression. As was the case with *crp*, the *himD* strain grew slower in most media, further complicating the interpretation of our results.

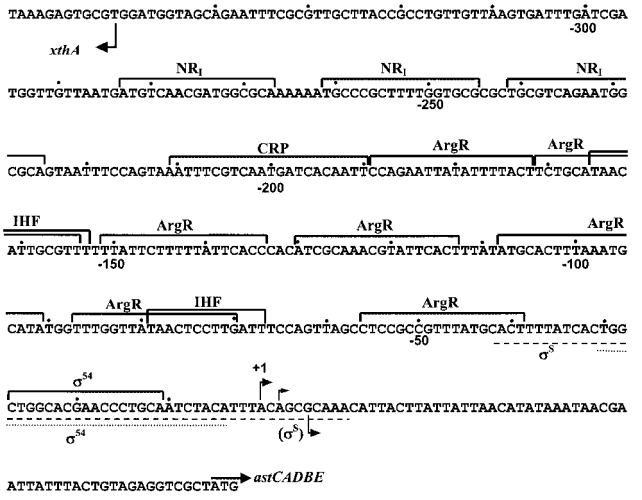

FIG. 6. The *ast* promoter of *E. coli*. The sequence of the *ast* promoter is shown together with the potential binding sites of various factors. Bent arrows, transcription start sites; straight arrow, start codon of *astC*. Dots mark every 10th nucleotide from the most upstream transcription initiation site (+1). The lines under the sequence indicate the extents of the footprints for the σ^S holoenzyme (dashed line) and the σ^N holoenzyme (dotted line).

The data presented here do not support an essential role for IHF in the regulation of the *ast* operon in *E. coli*.

Protein-DNA interactions at the *ast* promoter in vitro. Computer analysis and visual inspection of the promoter sequence revealed putative regulatory elements throughout the promoter region (Fig. 6). A σ^{54} site near the transcription start site and two closely spaced NR_I-binding sites, centered at −233 and −253, were easily recognizable. There are seven potential ArgR-binding sites between the σ^{54} and the NR_I sites and also a potential CRP-binding site just downstream from the enhancer sites. Two potential IHF sites, overlapping ArgR sites, were also discernible. To confirm the results concerning the factors that control *astCADBE* expression and to determine whether the potential binding sites actually bind proteins, we analyzed the interaction of purified proteins with the *ast* promoter region in vitro with DNase I footprinting.

(i) NR_I. NR_I bound at the two predicted sites and, at higher concentrations, at an additional site centered at −275 (Fig. 7). A number of hypersensitive sites were seen, usually at the ends of the binding sites; these hypersensitive sites are indicative of DNA bending by NR_I and have been observed at other NR_I-dependent promoters (see, for example, Fig. 2 of reference 7). Quantitative analysis of the gel showed that the concentration of NR_I required for half-maximal occupancy of the downstream site decreases from 35 to 13 nM upon phosphorylation by acetyl-phosphate. This concentration is 5 to 10 times higher than that reported for the *glnA* promoter of *E. coli* (7, 50), which means that the enhancer of the *ast* operon is weaker than the *glnALG* enhancer.

(ii) ArgR. The arginine repressor in its free form did not bind to the promoter (data not shown). ArgR with 5 mM arginine bound to an extended region from −20 to −120 (Fig. 8). At higher protein concentrations additional footprints were observed in regions −130 to −150 and −200 to −230. As with

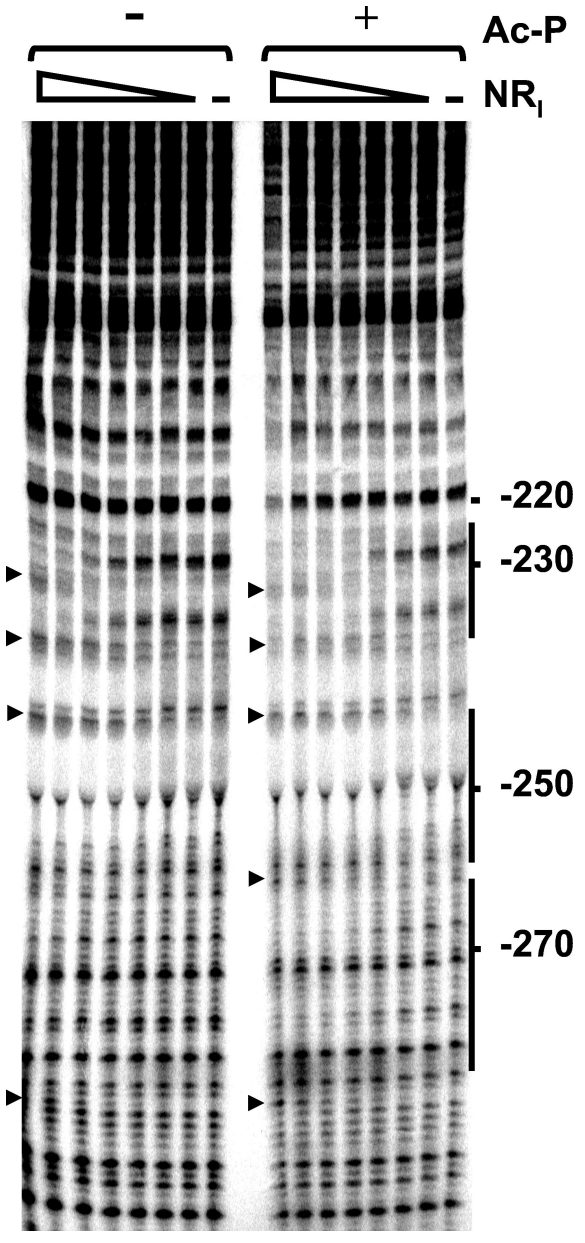

FIG. 7. Footprinting with NR_I. DNase I footprinting was performed on a fragment carrying the *ast* promoter region. The concentrations of the NR_I dimer used are 0, 4, 8, 16, 31, 62.5, 125, and 250 nM. Reactions were done in the presence or absence of acetyl-phosphate (Ac-P) as indicated. The position on the *ast* promoter sequence is also indicated. Solid lines, NR_I-binding sites; arrowheads, hypersensitive sites.

NR_I, sites of increased sensitivity to digestion denoted the bending caused by ArgR binding to the promoter. The size of the footprint in region −20 to −120 suggests that four ArgR-binding sites in the ArgR-promoter complex are occupied (Fig. 6). Assuming that one ArgR hexamer binds to two sites, as it does in other promoters (24), the binding of more than one ArgR hexamer would be required to give the observed footprint. An electrophoretic mobility shift assay showed multiple slowly migrating complexes (Fig. 9), suggesting that two or three ArgR hexamers might bind to the *ast* promoter simultaneously.

(iii) IHF. IHF protected two sites around −80 and −160 at concentrations higher than 20 nM (data not shown). These footprints overlap the ArgR-binding sites.

(iv) CRP. We could not detect specific binding of purified CRP to the *ast* promoter by DNase I footprinting with or without cAMP (not shown). We also did not observe binding in mobility shift assays, except at very high concentrations of CRP. With a 200-fold excess of CRP to DNA, we saw an abrupt transition from no binding to a shift where almost all the probe failed to enter the gel. We suspect that this is non-

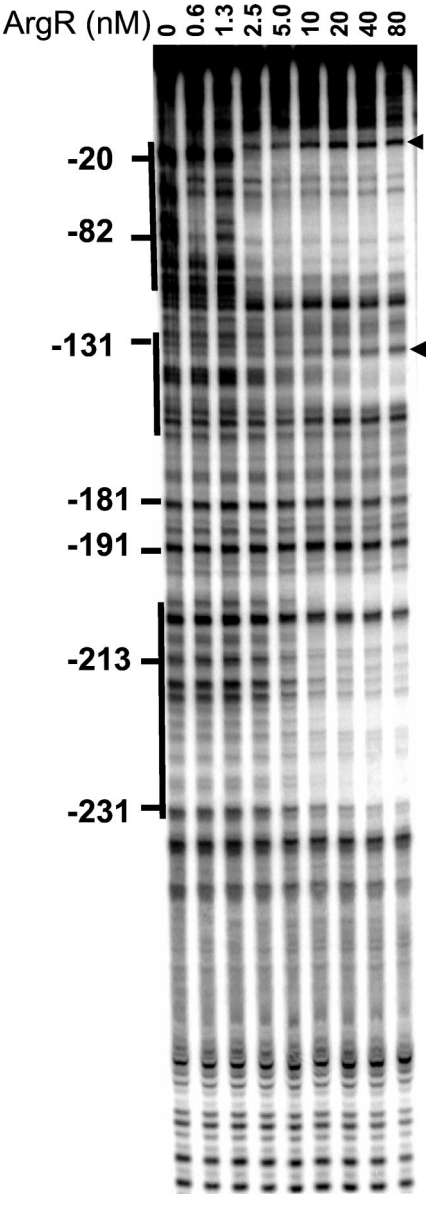

FIG. 8. Footprinting with ArgR. DNase I footprinting was performed on a fragment carrying the *ast* promoter with the indicated concentration of ArgR monomer and 5 mM arginine. The position on the *ast* promoter is shown. Solid lines, regions of protection; arrowheads, hypersensitive sites.

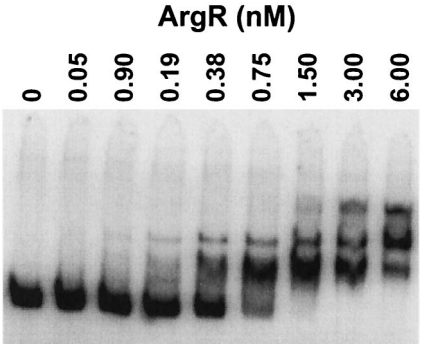

FIG. 9. Electrophoretic mobility shift assay with ArgR. A DNA fragment carrying the *ast* promoter region was incubated with the indicated concentration of ArgR monomer and 5 mM arginine prior to electrophoresis.

specific binding. Results with cells containing the *ast-lacZ* fusion suggested that CRP does not obviously contribute to *ast* expression in *E. coli*. This, together with the failure to observe specific binding, suggests that CRP does not directly control the *ast* operon. These results differ from genetic and biochemical results obtained with *S. enterica* serovar Typhimurium that suggest a direct role for CRP (23).

(v) RNA polymerase. DNase I footprinting showed that purified σ^{54} or σ^{S} together with core RNA polymerase bound the region around the sites of transcriptional initiation. The extent of the holoenzyme footprints is shown in Fig. 6.

Transcription from the *ast* promoters with purified components. The combination of in vivo and in vitro data presented thus far argues that the expression of the *ast* operon is regulated at two promoters. To further verify this conclusion, we analyzed transcription from these promoters with purified proteins. As shown in Fig. 10A, expression from the σ^{54} promoter was dependent on the presence of NR_I-phosphate. This transcription did not require the ArgR-arginine complex, although this complex enhanced transcription. IHF did not stimulate transcription and appeared to inhibit it when ArgR was also present.

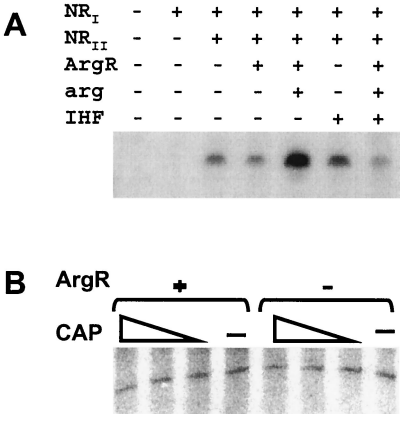

FIG. 10. In vitro transcription from the *ast* promoters. Single-round transcription experiments with purified components were performed from a supercoiled template with σ^{54} holoenzyme (A) or σ^{S} holoenzyme (B) and the indicated additions. The concentrations of CRP dimer used were 0, 200, 400, and 800 nM. The expected ~420-nucleotide transcript was obtained in both cases.

Transcription from the σ^{S}-dependent *ast* promoter required only σ^{S} holoenzyme (Fig. 10B). ArgR and CRP, alone or in combination, did not affect transcription (Fig. 10B). IHF also failed to affect transcription (not shown).

DISCUSSION

σ^{54} and σ^{S} are the major participants in *ast* operon expression. Mutants lacking both sigma factors have very low *ast* expression. There is some evidence for a σ^{70}-dependent promoter based on sequence analysis (just downstream of the σ^{54} and σ^{S} promoters) (see Fig. 3 of reference 15) and for the dependence of expression on σ^{70} based on results from a coupled in vitro transcription-translation system in crude extracts with a plasmid carrying an *ast-lacZ* fusion (5). However, our results clearly show that, if this σ^{70}-dependent promoter exists, it is not quantitatively important under the conditions we examined.

RNA polymerase holoenzyme competition. An interesting aspect of the regulation of the *ast* operon is the apparent competition between $E\sigma^{54}$ and $E\sigma^{S}$. Several lines of evidence indicate that each holoenzyme inhibits transcription by the other one. First, the proximity of the transcriptional start sites might preclude simultaneous binding and initiation. Second, during our primer extension analysis we did not encounter a condition in which we could detect transcripts from both promoters, which would indicate that, in every condition, the dominant holoenzyme takes over transcription of the operon entirely. Third, in some cases removal of a sigma factor responsible for transcription in a particular growth medium did not result in a significant change in the expression of the operon (Fig. 2 and 5; the *rpoS* mutant grown in glycerol-arginine medium and harvested in stationary phase). This indicates that only one promoter is utilized because of competition and not because the conditions do not allow for the utilization of the other promoter. Finally, in conditions in which expression could be initiated by only one form of the polymerase (e.g., $E\sigma^{S}$ in ammonia-containing media, which results in inactive $E\sigma^{54}$ since NR_I is largely unphosphorylated), deletion of the other sigma factor increases expression (Fig. 4). This idea of competition between the promoters in the *ast* operon has been previously postulated (15).

Stationary-phase induction and CRP. Our results show that the *ast* operon is induced in stationary phase, which confirms previous results. The *ast* operon was identified in a search for *E. coli* genes activated in response to conditioned medium (2) and another study looking for carbon starvation-induced genes (5). The apparent rationale for such control is the provision of citric acid cycle intermediates.

Stationary-phase expression initiates from the σ^{S} promoter, and we reconstituted σ^{S}-dependent transcription in vitro. The β-galactosidase results show that, as is the case in exponential phase, arginine stimulates expression but that exogenous arginine is not required for expression. This is most apparent for the *rpoN* mutant in glucose-containing media (compare β-galactosidase activity for cells grown in glucose-glutamine with that for cells grown in glucose-glutamine plus arginine in Fig. 4). This suggests that ArgR might also be involved in the

regulation of the σ^S promoter, perhaps by facilitating an interaction between $E\sigma^S$ and another DNA-bound transcription factor. Despite the effect of the carbon source on the expression of *astCADBE*, we could not demonstrate a clear involvement of CRP in vivo or in vitro. This is in contrast with what is found for *S. enterica* serovar Typhimurium, where CRP seems to be essential for stationary-phase expression (23), but is in agreement with results for *E. coli* arguing against a role of cAMP in the regulation of an *ast-lacZ* fusion (5). The basis for the difference in CRP responsiveness is not clear. An *E. coli* site with significant homology to the CRP consensus sequence can be found at the same position as the *S. enterica* serovar Typhimurium site. The sites in the two species differ by only 3 bp, with the *S. enterica* serovar Typhimurium site being closer to the consensus by only 1 bp. Unexpectedly though, while binding to the site on the *S. enterica* serovar Typhimurium promoter can be demonstrated in vitro (23), we could not obtain CRP binding to the *E. coli* promoter by mobility shift or DNase I footprinting.

Our results do not exclude the possibility that there is a binding site for an unidentified activator and that ArgR enhances its ability to activate transcription. It is known that stationary-phase induction of the *ast* operon is complex. In strain MC1061, this induction is mediated, at least in part, through indole (47). However, while indole production by W3110 has been reported previously (21), some W3110 strains contain an insertion of an IS *5* element in the *tnaB* gene of the *tnaAB* operon (20) (*tnaA* codes for tryptophanase, the indole-producing enzyme [43]), and our W3110 strain is indole negative based on a standard microbiological test (H. Kasbarian and L. Reitzer, unpublished results). We tested induction by indole in 0.5× LB medium (the $E\sigma^{54}$ promoter is inactive) and found that *ast-lacZ* expression in mid-exponential phase increased from 1,300 to 5,700 nmol min^{-1} mg of $protein^{-1}$. Therefore, although our W3110 does not produce indole, it retains the factors necessary to respond to its presence. Nonetheless, there is an additional unidentified factor that activates the *ast* operon and other operons in stationary phase (47), and ArgR could potentially enhance its ability to stimulate transcription.

Carbon source control of *ast* expression. There is lower *ast* operon expression in exponential phase with glycerol instead of glucose as the carbon source (Fig. 3). This effect appears to be both CRP and σ^{54} dependent (Fig. 4 and 5). It has been reported that CRP-cAMP can interact directly with σ^{54} and inhibit its ability to activate transcription (45, 48). This could explain the carbon source control.

General induction by nitrogen limitation. The *astCADBE* operon in *E. coli* codes for the enzymes of the AST pathway, which are necessary for the utilization of arginine as a nitrogen source (40). The AST pathway produces the ammonia required for growth in nitrogen-limiting minimal media and also produces glutamate. The levels of the AST enzymes are increased in nitrogen-limiting conditions, as would be expected given the function of the AST pathway (40). This aspect of the regulation of the operon is mediated through σ^{54} and NR_I. The involvement of NR_I has been seen before through measurements of AST enzyme levels (40) and gene arrays (52). Here, we complement these results by reconstituting the activation of the *ast* promoter by NR_I in vitro.

ArgR and arginine-specific enhancement during nitrogen-limited growth. Most NR_I-dependent Ntr genes are expressed in nitrogen-limiting conditions regardless of the specific nitrogen source. The *ast* operon is different: arginine as a nitrogen source enhances expression (40; this study). Based on the in vivo and in vitro results of our analysis, ArgR mediates this specific induction. The ArgR-dependent induction is not essential for expression, either in vivo or in vitro. For several reasons it seems that the role of ArgR is to facilitate the interaction between NR_I and $E\sigma^{54}$. First, the NR_I-binding sites are centered at −233 and −253 from the start site of transcription, a distance significantly greater than that in any other known NR_I-dependent promoter. This distance might make direct contact between the promoter-bound polymerase and the enhancer-bound NR_I less likely. Second, the ArgR-binding sites are positioned appropriately to facilitate an RNA polymerase-NR_I interaction. Third, this proposal is consistent with the known properties of ArgR. The binding of an ArgR hexamer to two ArgR-binding sites causes the DNA to bend by 70° (44). If more than one hexamer binds to the *ast* promoter, as implied by the mobility shift and footprinting results, they could cause a bend closer to 180°, which would be comparable to the bend caused by IHF binding (34). The *E. coli ast* operon differs in its regulation from the *S. enterica* serovar Typhimurium operon, which appears to absolutely require ArgR for expression. It is not clear why this role of ArgR is essential in *S. enterica* serovar Typhimurium but not in *E. coli*.

It is not uncommon for a DNA-bending protein to promote an interaction between $E\sigma^{54}$ and an activator, and IHF is frequently the DNA-bending protein (8, 11, 18, 39, 49). However, our results do not suggest an effect of the *himD* mutation in the regulation of *astCADBE* in vivo. Furthermore, the IHF footprint was weak, and purified IHF had no positive effect on transcription in vitro. These results suggest that IHF does not positively contribute to *ast* operon expression. The IHF footprint could be an artifactual result of the AT richness of the *ast* promoter, which is a feature of both ArgR-binding sites and σ^{54}-dependent promoters (IHF-binding sites are AT rich). It is also possible that, earlier in bacterial evolution, IHF was involved in *ast* operon expression and that ArgR preempted this function. In this case, a residual IHF-binding site might still remain.

Arginine-responsive transcription factors commonly participate in the regulation of arginine synthesis or degradation or both in many diverse bacterial species. The ArgR protein of *Bacillus licheniformis* activates the *arcABCD* gene cluster, which encodes the enzymes of the arginine deiminase pathway (26). The ArgR homologue of *Bacillus subtilis*, AhrC, activates the *rocABC* and *rocDEF* operons of arginine and ornithine catabolism by a mechanism which, apparently, involves direct protein-protein interaction with RocR, a σ^{54}-dependent activator (16, 27). Overexpression of the *argR* gene of *Streptomyces clavuligerus* causes repression of an arginine biosynthetic enzyme and activation of a catabolic one (35). Finally, the ArgR of *Pseudomonas aeruginosa*, a transcriptional activator of the AraC/XylS family unrelated to the ArgR of the enteric bacteria and the bacilli, activates the arginine anabolic genes and represses the arginine catabolic genes (32).

The functions of nonspecific ArgR-independent induction

and ArgR-dependent superinduction. Regardless of the promoter and sigma factor used, there is ArgR-independent expression overlaid with ArgR-dependent expression (or superinduction). Therefore, the function of ArgR-dependent control must be considered within the context of general control mechanisms and their functions. It has been proposed that a major function of the σ^{54}-dependent Ntr response is nitrogen scavenging (52). This is reasonable based on the nonspecific (nitrogen source-independent) induction of Ntr genes. Perhaps it is even surprising that all known genes induced by nitrogen limitation in *E. coli* do not require specific induction, even though specific regulators sometimes exist (e.g., for the *ast* and *gab* operons). Nonspecific induction might reflect the environment, i.e., nitrogen limitation may frequently involve a number of nitrogen sources at low concentration. However, a scavenging function does not account for why *E. coli* scavenges a limited number of nitrogen-containing compounds and why *E. coli* has so few σ^{54}-dependent catabolic pathways for nitrogen source utilization. Very few nitrogen sources are degraded by σ^{54}-dependent enzymes, and these include arginine, γ-aminobutyrate (via Nac) (52; S. Ruback, A. Kiupakis, and L. Reitzer, unpublished results), and cytosine (52). There is actually a good reason for a σ^{54}-dependent pathway of arginine catabolism. It can be calculated from the composition of *E. coli* (31) that arginine in protein (281 μmol/g dry weight) constitutes 10.9% of the total cellular nitrogen (1,124 μmol of nitrogen in arginine compared to 10,283 μmol of nitrogen/g of *E. coli*), which is surprisingly large. (Guanine nucleotides comprise 11.1%, and adenine nucleotides comprise 9.2%, of total nitrogen, but no other single macromolecular monomer contains as much nitrogen.) Assuming that other organisms have similar nitrogen contents, then a major source of organic nitrogen from recycled organisms would be arginine. Therefore, an arginine catabolic pathway would be important for nitrogen acquisition, which accounts for general induction by nitrogen limitation. An ArgR-dependent superinduction would be reasonable if there were environments in which arginine was a major component. Some organisms, such as *Saccharomyces cerevisiae*, have vacuoles that contain arginine. The lysis of organisms such as this could create an arginine-rich milieu.

The same pattern of nonspecific induction followed by a possible ArgR-dependent superinduction may also exist for the σ^{S}-dependent promoter. The rationale for such regulation might be similar to that for the two layers of control for the σ^{54}-dependent promoter. A nonspecific induction during stationary-phase metabolism would provide citric acid cycle intermediates. The superinduction would then occur only in arginine-rich environments, and this might require ArgR with an as yet unidentified activator.

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REFERENCES

1. **Aiba, H., S. Adhya, and B. de Crombrugghe.** 1981. Evidence for two functional gal promoters in intact *Escherichia coli* cells. J. Biol. Chem. **256:**11905–11910.
2. **Baca-DeLancey, R. R., M. M. South, X. Ding, and P. N. Rather.** 1999. *Escherichia coli* genes regulated by cell-to-cell signaling. Proc. Natl. Acad. Sci. USA. **96:**4610–4614.
3. **Becker, G., E. Klauck, and R. Hengge-Aronis.** 1999. Regulation of RpoS proteolysis in *Escherichia coli*: the response regulator RssB is a recognition factor that interacts with the turnover element in RpoS. Proc. Natl. Acad. Sci. USA **96:**6439–6444.
4. **Bender, R. A.** 1991. The role of the NAC protein in the nitrogen regulation of *Klebsiella aerogenes*. Mol. Microbiol. **5:**2575–2580.
5. **Blum, P. H., S. B. Jovanovich, M. P. McCann, J. E. Schultz, S. A. Lesley, R. R. Burgess, and A. Matin.** 1990. Cloning and in vivo and in vitro regulation of cyclic AMP-dependent carbon starvation genes from *Escherichia coli*. J. Bacteriol. **172:**3813–3820.
6. **Boone, T., and G. Wilcox.** 1978. A rapid high-yield purification procedure for the cyclic adenosine 3′,5′-monophosphate receptor protein from *Escherichia coli*. Biochim. Biophys. Acta **541:**528–534.
7. **Chen, P., and L. J. Reitzer.** 1995. Active contribution of two domains to cooperative DNA binding of the enhancer-binding protein nitrogen regulator I (NtrC) of *Escherichia coli*: stimulation by phosphorylation and the binding of ATP. J. Bacteriol. **177:**2490–2496.
8. **Claverie-Martin, F., and B. Magasanik.** 1991. Role of integration host factor in the regulation of the *glnHp2* promoter of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **88:**1631–1635.
9. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
10. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn *5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
11. **Dworkin, J., G. Jovanovic, and P. Model.** 1997. Role of upstream activation sequences and integration host factor in transcriptional activation by the constitutively active prokaryotic enhancer-binding protein PspF. J. Mol. Biol. **273:**377–388.
12. **Eilen, E., C. Pampeno, and J. S. Krakow.** 1978. Production and properties of the α core derived from the cyclic adenosine monophosphate receptor protein of *Escherichia coli*. Biochemistry **17:**2469–2473.
13. **Elliott, T.** 1992. A method for constructing single-copy *lac* fusions in *Salmonella typhimurium* and its application to the *hemA-prfA* operon. J. Bacteriol. **174:**245–253.
14. **Elliott, T., and E. P. Geiduschek.** 1984. Defining a bacteriophage T4 late promoter: absence of a "−35"region. Cell **36:**211–219.
15. **Fraley, C. D., J. H. Kim, M. P. McCann, and A. Matin.** 1998. The *Escherichia coli* starvation gene *cstC* is involved in amino acid catabolism. J. Bacteriol. **180:**4287–4290.
16. **Gardan, R., G. Rapoport, and M. Debarbouille.** 1997. Role of the transcriptional activator RocR in the arginine-degradation pathway of *Bacillus subtilis*. Mol. Microbiol. **24:**825–837.
17. **Heyduk, T., and J. C. Lee.** 1989. *Escherichia coli* cAMP receptor protein: evidence for three protein conformational states with different promoter binding affinities. Biochemistry **28:**6914–6924.
18. **Hoover, T. R., E. Santero, S. Porter, and S. Kustu.** 1990. The integration host factor stimulates interaction of RNA polymerase with NIFA, the transcriptional activator for nitrogen fixation operons. Cell **63:**11–22.
19. **Hunt, T. P., and B. Magasanik.** 1985. Transcription of *glnA* by purified *Escherichia coli* components: core RNA polymerase and the products of *glnF*, glnG, and *glnL*. Proc. Natl. Acad. Sci. USA **82:**8453–8457.
20. **Kamath, A. V., K. Gish, and C. Yanofsky.** 1994. A copy of insertion element IS *5* is present within *tnaB* in the Kohara library of *Escherichia coli* W3110. J. Bacteriol. **176:**1546–1547.
21. **Kell, R. G. D. B.** 1993. Rapid and quantitative analysis of bioprocesses using pyrolysis mass spectrometry and neural networks: application to indole production. Anal. Chim. Acta **279:**17–26.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
23. **Lu, C. D., and A. T. Abdelal.** 1999. Role of ArgR in activation of the *ast* operon, encoding enzymes of the arginine succinyltransferase pathway in *Salmonella typhimurium*. J. Bacteriol. **181:**1934–1938.
24. **Maas, W. K.** 1994. The arginine repressor of *Escherichia coli*. Microbiol. Rev. **58:**631–640.
25. **Magasanik, B.** 1996. Regulation of nitrogen utilization, p. 1344–1356. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
26. **Maghnouj, A., T. F. de Sousa Cabral, V. Stalon, and C. Vander Wauven.** 1998. The *arcABDC* gene cluster, encoding the arginine deiminase pathway

of *Bacillus licheniformis*, and its activation by the arginine repressor ArgR. J. Bacteriol. **180:**6468–6475.
27. **Miller, C. M., S. Baumberg, and P. G. Stockley.** 1997. Operator interactions by the *Bacillus subtilis* arginine repressor/activator, AhrC: novel positioning and DNA-mediated assembly of a transcriptional activator at catabolic sites. Mol. Microbiol. **26:**37–48.
28. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Moore, J. B., S. P. Shiau, and L. J. Reitzer.** 1993. Alterations of highly conserved residues in the regulatory domain of nitrogen regulator I (NtrC) of *Escherichia coli*. J. Bacteriol. **175:**2692–2701.
30. **Nash, H. A., C. A. Robertson, E. Flamm, R. A. Weisberg, and H. I. Miller.** 1987. Overproduction of *Escherichia coli* integration host factor, a protein with nonidentical subunits. J. Bacteriol. **169:**4124–4127.
31. **Neidhardt, F. C., and H. E. Umbarger.** 1996. Chemical composition of *Escherichia coli*, p. 13–16. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington D.C.
32. **Park, S. M., C. D. Lu, and A. T. Abdelal.** 1997. Cloning and characterization of *argR*, a gene that participates in regulation of arginine biosynthesis and catabolism in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **179:**5300–5308.
33. **Reitzer, L. J.** 1996. Sources of nitrogen and their utilization, p. 380–390. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D.C.
34. **Rice, P. A., S. Yang, K. Mizuuchi, and H. A. Nash.** 1996. Crystal structure of an IHF-DNA complex: a protein-induced DNA U-turn. Cell **87:**1295–1306.
35. **Rodriguez-Garcia, A., M. Ludovice, J. F. Martin, and P. Liras.** 1997. Arginine boxes and the *argR* gene in *Streptomyces clavuligerus*: evidence for a clear regulation of the arginine pathway. Mol. Microbiol. **25:**219–228.
36. **Rothstein, D. M., G. Pahel, B. Tyler, and B. Magasanik.** 1980. Regulation of expression from the *glnA* promoter of *Escherichia coli* in the absence of glutamine synthetase. Proc. Natl. Acad. Sci. USA **77:**7372–7376.
37. **Sabourin, D., and J. Beckwith.** 1975. Deletion of the *Escherichia coli crp* gene. J. Bacteriol. **122:**338–340.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Santero, E., T. R. Hoover, A. K. North, D. K. Berger, S. C. Porter, and S. Kustu.** 1992. Role of integration host factor in stimulating transcription from the sigma 54-dependent *nifH* promoter. J. Mol. Biol. **227:**602–620.
40. **Schneider, B. L., A. K. Kiupakis, and L. J. Reitzer.** 1998. Arginine catabolism and the arginine succinyltransferase pathway in *Escherichia coli*. J. Bacteriol. **180:**4278–4286.
41. **Schneider, B. L., S. P. Shiau, and L. J. Reitzer.** 1991. Role of multiple environmental stimuli in control of transcription from a nitrogen-regulated promoter in *Escherichia coli* with weak or no activator-binding sites. J. Bacteriol. **173:**6355–6363.
42. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
43. **Snell, E. E.** 1975. Tryptophanase: structure, catalytic activities, and mechanism of action. Adv. Enzymol. Relat. Areas Mol. Biol. **42:**287–333.
44. **Tian, G., D. Lim, J. Carey, and W. K. Maas.** 1992. Binding of the arginine repressor of *Escherichia coli* K12 to its operator sites. J. Mol. Biol. **226:**387–397.
45. **Tian, Z. X., Q. S. Li, M. Buck, A. Kolb, and Y. P. Wang.** 2001. The CRP-cAMP complex and downregulation of the *glnAp2* promoter provides a novel regulatory linkage between carbon metabolism and nitrogen assimilation in *Escherichia coli*. Mol. Microbiol. **41:**911–924.
46. **Ueno-Nishio, S., K. C. Backman, and B. Magasanik.** 1983. Regulation at the *glnL*-operator-promoter of the complex *glnALG* operon of *Escherichia coli*. J. Bacteriol. **153:**1247–1251.
47. **Wang, D., X. Ding, and P. N. Rather.** 2001. Indole can act as an extracellular signal in *Escherichia coli*. J. Bacteriol. **183:**4210–4216.
48. **Wang, Y. P., A. Kolb, M. Buck, J. Wen, F. O'Gara, and H. Buc.** 1998. CRP interacts with promoter-bound sigma54 RNA polymerase and blocks transcriptional activation of the *dctA* promoter. EMBO J. **17:**786–796.
49. **Wassem, R., E. M. De Souza, M. G. Yates, F. D. Pedrosa, and M. Buck.** 2000. Two roles for integration host factor at an enhancer-dependent *nifA* promoter. Mol. Microbiol. **35:**756–764.
50. **Weiss, V., F. Claverie-Martin, and B. Magasanik.** 1992. Phosphorylation of nitrogen regulator I of *Escherichia coli* induces strong cooperative binding to DNA essential for activation of transcription. Proc. Natl. Acad. Sci. USA **89:**5088–5092. (Erratum, **89:**8856.)
51. **Zhang, X. P., A. Gunasekera, Y. W. Ebright, and R. H. Ebright.** 1991. Derivatives of CAP having no solvent-accessible cysteine residues, or having a unique solvent-accessible cysteine residue at amino acid 2 of the helix-turn-helix motif. J. Biomol. Struct. Dyn. **9:**463–473.
52. **Zimmer, D. P., E. Soupene, H. L. Lee, V. F. Wendisch, A. B. Khodursky, B. J. Peter, R. A. Bender, and S. Kustu.** 2000. Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: scavenging as a defense against nitrogen limitation. Proc. Natl. Acad. Sci. USA **97:**14674–14679.