JOURNAL OF BACTERIOLOGY, Mar. 1999, p. 1934–1938 Vol. 181, No. 6
0021-9193/99/$04.00+0

Role of ArgR in Activation of the *ast* Operon, Encoding Enzymes of the Arginine Succinyltransferase Pathway in *Salmonella typhimurium*

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Received 16 September 1998/Accepted 5 January 1999

The *ast* operon, encoding enzymes of the arginine succinyltransferase (AST) pathway, was cloned from *Salmonella typhimurium*, and the nucleotide sequence for the upstream flanking region was determined. The control region contains several regulatory consensus sequences, including binding sites for NtrC, cyclic AMP receptor protein (CRP), and ArgR. The results of DNase I footprintings and gel retardation experiments confirm binding of these regulatory proteins to the identified sites. Exogenous arginine induced AST under nitrogen-limiting conditions, and this induction was abolished in an *argR* derivative. AST was also induced under carbon starvation conditions; this induction required functional CRP as well as functional ArgR. The combined data are consistent with the hypothesis that binding of one or more ArgR molecules to a region between the upstream binding sites for NtrC and CRP and two putative promoters plays a pivotal role in modulating expression of the *ast* operon in response to nitrogen or carbon limitation.

The arginine succinyltransferase (AST) pathway, which converts arginine to glutamate, has been long considered the major route for aerobic utilization of arginine as a source of carbon, nitrogen, and energy by *Pseudomonas aeruginosa* (7, 28). Characterization of the *aru* operon, encoding enzymes of this pathway in *P. aeruginosa*, led to the identification of the corresponding *ast* operon from the *Escherichia coli* genome sequence (10). Recent studies have shown that the AST pathway, rather than the arginine decarboxylase pathway, is the major pathway for utilization of arginine as a nitrogen source by *E. coli* (23). Interestingly, this pathway is also important for carbon starvation survival, such that one of the *ast* genes of *E. coli* was initially identified as a starvation gene, *cstC* (1, 3).

Computer analysis of the nucleotide sequence of the region upstream of the *ast* operon in *E. coli* identified a putative σ^{54} consensus sequence and two putative NtrC binding sites; such sequences are consistent with the observed nitrogen regulation of the operon (3, 23). Studies by Fraley et al. (3) also indicate the presence of a σ^{S} promoter that appears to compete with the σ^{54} promoter to match expression to cellular needs.

We have reported recently (20) that the arginine regulatory protein of *P. aeruginosa* is required for induction of the AST pathway by exogenous arginine. While the structure and function of the arginine regulatory proteins of *P. aeruginosa* and *Salmonella typhimurium* differ significantly (14, 20, 21), an early finding by Kustu (12) indicated that an *argR* derivative of *S. typhimurium* is impaired in utilization of arginine as a nitrogen source. Studies by Kustu et al. (13) also indicated that arginine degradation in this organism is under nitrogen control. Assuming that the recently identified *ast* operon of *E. coli* (10) would have a homologue in the closely related *S. typhimurium*, we initiated an investigation of the possible role of ArgR of *S. typhimurium* in expression of the *ast* operon.

(A preliminary report of this work has been presented previously [16].)

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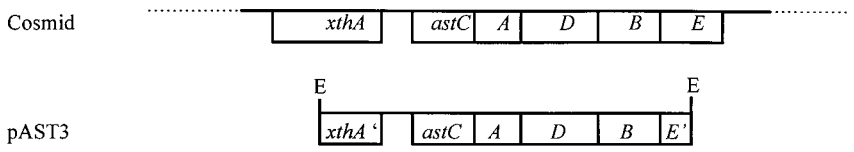
Cloning of the *ast* operon and sequence features of the upstream flanking region. A DNA fragment covering the first 500 bp of the *astC* structural gene of *E. coli* was amplified by PCR from *E. coli* K-12 chromosomal DNA. This DNA fragment was then purified, labeled by the Genius system (Boehringer), and used in colony hybridization for screening of a cosmid library of *S. typhimurium* constructed in this laboratory. Several positive clones were identified, and a 6.5-kb *Eco*RI fragment from one of these cosmids was further subcloned into the *Eco*RI site of pUC18, as shown in Fig. 1. The chromosomal insert of the resulting plasmid (pAST3 [Fig. 1]) was partially sequenced, and a homology search indicated that it contains most of the *astCABDE* operon and an upstream flanking region of 470 bp. The *ast* operon structure of *S. typhimurium* was found to be identical to its counterpart in *E. coli* (10). Furthermore, the *xthA* gene was also found upstream of the *ast* operon, as is the case in *E. coli* (GenBank accession no. D90818).

The upstream region flanking the *ast* operon was amplified by PCR from plasmid pAST3. Restriction sites of *Bam*HI were introduced into a pair of primers for PCR, and the PCR product of a 490-bp DNA fragment was cloned into the *Bam*HI site of vector pUC19. The resulting plasmid was designated pAST101; the nucleotide sequence of the chromosomal insert in this plasmid was confirmed to be identical to that in pAST3 and is shown in Fig. 1. Certain features noted in the upstream sequence in *E. coli* (3)—a putative σ^{54} promoter (18), two potential NtrC binding sites (18), and a putative integration host factor (IHF) binding site (4)—are present at the corresponding locations in the *S. typhimurium* sequence. However, there is little homology between the two sequences in the regions identified as cyclic AMP receptor protein (CRP) binding sites in the *E. coli* sequence (3). The consensus sequence of the CRP binding site, 5′-AAATGTGATCTAGATCACATT T-3′, consists of two 11-bp half sites organized as inverted repeats that accommodate CRP dimer (22). The *S. typhimurium* sequence (Fig. 1) contains a sequence downstream of the NtrC sites that appears to be a good candidate for a CRP site. The first half of the site proposed here has poor homology to the consensus sequence (4 of 11 bp) but the second half exhibits excellent homology to the consensus (10 of 11 bp). Six

a.



b.

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GGATCCAGAGACAAATTTCATAGTCACTGCCACTTTGCAAGGTGAATCGTGTTGGGATGGTAACAGAAAT    70
BamHI          <-xthA

TTTACCGGGTGACTGCCACTCATCGTCAGATTCGGCCAATGGGAAGCGTAGTGGTGTTTTCTATAGCGCG   140

      NtrC I          NtrC II                    CRP
AAAATGGCACGGTTTGCGTGCAGATGCGCCAGAACGGGGCGGTAATTTCCAGTAAATTTCGTATAAGATC   210
                                                                  Sau3A

                  ARG box I       ARG box II
ACAATTCCAGAATTATATTTGGCAACTGAATAATATTTCGCTTTTTCCTCTTTTTAGCCACCGATTATGC   280

 ARG box III     ARG box IV                    ARG box V
GGAAATATTCACTTTTCATGCAGTAAAAGTGAATATCGCTACGCTGTAACCCATTGATTTACCGTTTATC   350
                                                 IHF

 ARG box VI          σ54
ACCTCTGTTTATGCAATGTTAACGCTGGCTGGCACGAATGCTGCAATCTACATTTACAGCGCAAACACTC   420

                                  S.D.
ATATTAATTAACATATAAATAACTTTATTTTTACCAAAAGAGGTCGCTATGTCTCTGTCAGTTGGATCC   489
                                  astC->                  BamHI
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FIG. 1. Nucleotide sequence of the *ast* regulatory region of *S. typhimurium*. (a) Schematic drawing of the structure of the *ast* operon in a cosmid and one of the subclones, pAST3. (b) Nucleotide sequence of the chromosomal insert in pAST101. The proposed σ^{54} promoter region and the putative binding sites for NtrC, ArgR, CRP, and IHF are labeled. The DNA regions protected in DNase I experiments are shown in boldface italic letters. The initiation codons for the *astC* and *xthA* genes and the *Bam*HI and *Sau*3A restriction sites are also labeled. The Shine-Dalgarno sequence for *astC* is overlined and labeled S.D. This *Bam*HI fragment is cloned into pUC19 in such an orientation that *Hin*dIII and *Sal*I are at the 5′ end, and *Sma*I and *Eco*RI are at the 3′ end of the sequence shown here.

putative ArgR boxes can be also deduced, albeit with varying degrees of homology to the consensus sequence (5′-AATGA ATAATTATTCATT-3′ [29]). Our previous studies with ArgR of *S. typhimurium* indicate that it is a hexamer of identical 17,000 M_r subunits and that each hexamer binds to two such ARG boxes (14).

Binding of ArgR to the regulatory region of the *ast* operon. The purified 490-bp *Bam*HI fragment of pAST101, which contains the entire *ast* regulatory region, was labeled with [α-^{32}P]dGTP by using the Klenow fragment. The labeled fragment was digested by *Sau*3A to generate two end-labeled fragments; one of them is 210 bp and carries the two putative NtrC binding sites, and the other is 280 bp and carries the putative ArgR binding sites (Fig. 1). These two labeled fragments were used in gel retardation experiments employing a homogeneous ArgR preparation that was purified as previously described (14). The results (Fig. 2, top) show that ArgR interacts specifically with the 280-bp fragment carrying the putative ArgR binding sites. A plot of the percentage of bound DNA against the concentration of ArgR yields an apparent dissociation constant of 5.0 pM.

DNase I footprinting experiments were carried out as described previously (14), employing a ^{32}P-labeled *Hin*dIII/*Eco*RI fragment from pAST101 (Fig. 1b). For strand-specific detection, the resulting radioactive probe was subjected to *Sma*I digestion for the bottom strand and *Sal*I digestion for the top strand. The results (Fig. 3) show that binding of ArgR protects a 90-bp region on the top strand at lower concentrations of ArgR and that this protection is extended at higher ArgR concentrations to 140 bp. As shown in Fig. 1, two ArgR binding sites (corresponding to four ARG boxes) reside in the first 90-bp protected region, and one additional site resides in the extended downstream region.

NtrC binding sites. DNase I footprinting experiments were carried out, employing a purified MBP-NtrC fusion protein (11) that was generously provided by S. Kustu (Berkeley, Calif.). The results (Fig. 3) show that binding of NtrC protects a 55-bp region from nuclease digestion on the bottom strand, with the two NtrC binding sites, predicted by computer analysis, in the center of the protected region.

CRP binding. Computer analysis of the nucleotide sequence of the control region in *S. typhimurium* led to the identification of a potential CRP site centered at nucleotide 205 (Fig. 1). This site is at a different location from the potential sites proposed for the *E. coli* operon (3). Gel retardation experiments were carried out, employing a DNA fragment carrying the entire 490-bp regulatory region and the CRP protein of *E. coli* (purified according to reference 31; a gift from P. C. Tai). The results (Fig. 2, bottom) show that CRP specifically binds to the regulatory region. Cleavage by *Sau*3A within the identified site (Fig. 1) produces two DNA fragments that lost the capacity to bind CRP in gel retardation experiments (Fig. 2, bottom).

Effects of *argR* and *ntrB*(Con) on AST activity. The effect of exogenous arginine on the expression of the *ast* operon was

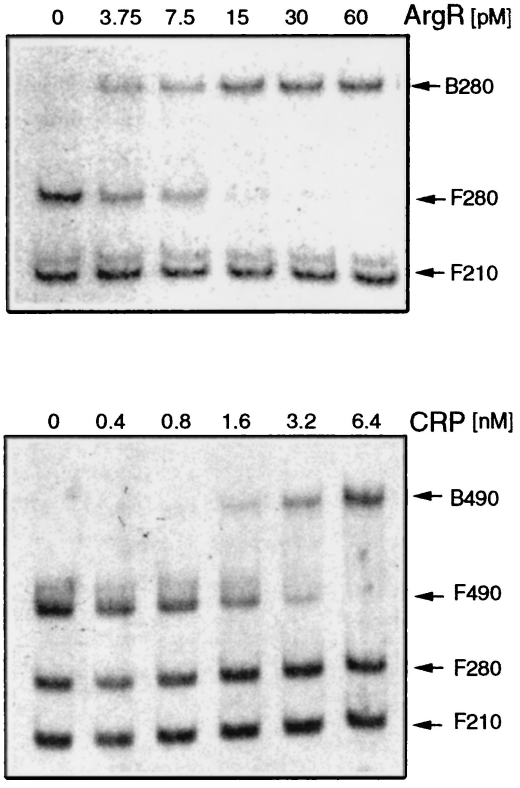

FIG. 2. Gel retardation assays with purified ArgR (top panel) and purified CRP (bottom panel). The reactions with purified ArgR (14) and CRP (24) were carried out as described previously. Radioactively labeled probes (0.1 pM) were allowed to interact with different protein concentrations, as indicated. Labeled fragments are as follows: F490 and B490, free and bound forms of the 490-bp *Bam*HI fragment; F280 and B280, free and bound forms of the 280-bp *Sau*3A/*Bam*HI fragment; F210, free probe of the 210-bp *Bam*HI/*Sau*3A fragment.

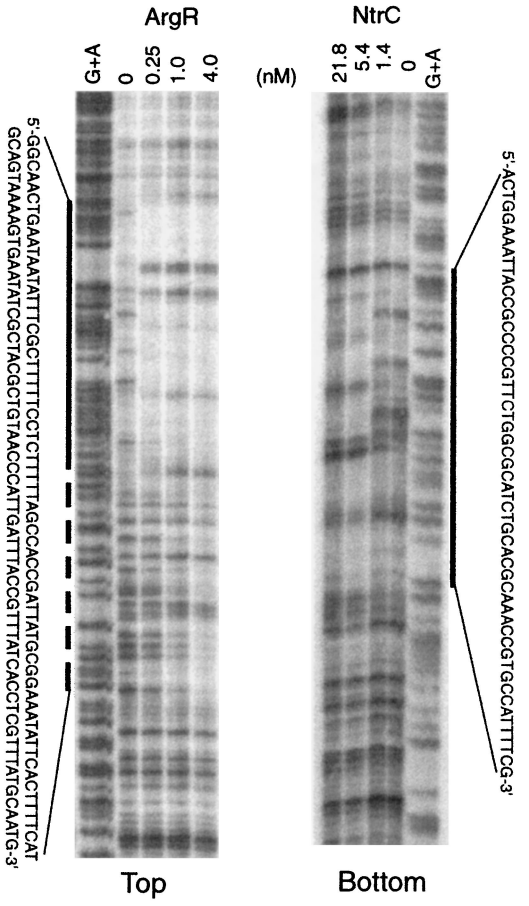

FIG. 3. DNase I footprinting with purified ArgR and NtrC. The reactions with ArgR (14) and MBP-NtrC (11) were carried out as described previously. DNA probes (0.17 nM) labeled at the top and bottom strands were used in the reactions with ArgR and NtrC, respectively. The concentrations of ArgR and NtrC used in reactions are indicated on the top of each lane. The corresponding G+A Maxam-Gilbert sequencing ladders (19) are also labeled. A solid line at lower concentrations and a dashed line for the extended region at higher concentrations mark the regions protected by ArgR. A solid line marks the region protected by NtrC. Nucleotide sequences for the protected regions indicated here correspond to boldface italic letters in Fig. 1.

monitored by measurement of AST, the first enzyme of the AST pathway. Cultures of wild-type *S. typhimurium* and an *argR* derivative (15) were grown in glucose minimal medium (6) with either glutamate or glutamate and arginine as the source(s) of nitrogen. Under these conditions, nitrogen is limiting and the doubling time (270 to 500 min) is much longer than that obtained with excess ammonia (45 min). The results (Table 1) show that exogenous arginine induces AST activity by 7.3-fold and that this induction is abolished in the *argR*::Tn*10* derivative.

AST activity was also measured in an *ntrB*(Con) derivative (strain SK3003); this constitutive mutation causes overexpression of nitrogen-regulated genes regardless of nitrogen level (9) and results in faster growth under nitrogen-limiting conditions. The results (Table 1) show that *ntrB*(Con), grown with glutamate as the sole nitrogen source, has 13.4-fold-higher AST activity than the wild type. This higher activity reflects the higher concentration of functional NtrC in this strain. Exogenous arginine is still able to cause an additional fourfold induction. Introduction of the *argR*::Tn*10* allele in the *ntrB*(Con) background again abolishes induction by arginine when cells are grown with both glutamate and arginine. AST activity is somewhat higher when the *ntrB*(Con) *argR* mutant derivative is grown with arginine as the sole nitrogen source but is still 5.6-fold lower than in the *ntrB*(Con) *argR*$^+$ background. This somewhat higher level likely reflects a higher concentration of functional NtrC as a result of the much longer doubling time (300 min) observed under these conditions.

These results indicate that ArgR is essential for arginine induction of the AST pathway and that this induction is likely mediated at an NtrC-dependent promoter.

Both *argR* and *crp* genes are essential for induction of the *ast* operon under carbon starvation. AST activity was measured in wild-type *S. typhimurium* and its *argR* and *crp* derivatives in the presence of excess ammonia and under conditions of glucose excess and limitation. The results (Table 2) show that the wild-type strain has a negligible level of AST activity in the presence of excess ammonia and glucose, regardless of the absence or presence or arginine. In contrast, an elevated level of AST activity was observed following depletion of a limiting amount of glucose. These results establish that carbon starvation induces AST activity in the presence of excess ammonia.

Inactivation of *crp* by Tn*10* insertion abolishes induction by carbon starvation, regardless of the absence or presence of exogenous arginine. Interestingly, inactivation of *argR* also abolishes induction by carbon starvation. Thus, the presence of

TABLE 1. Effects of exogenous arginine on induction of AST in wild-type *S. typhimurium* and its *argR*::Tn*10* and *ntrB*(Con) derivatives

Genotype	Nitrogen source[a]	Doubling time (min)	Sp act (nmol/mg/min)[b]
Wild type	Glu	270	11 (1)
	Glu+Arg	270	80 (3)
argR::Tn*10*	Glu	450	11 (2)
	Glu+Arg	500	9 (1)
ntrB(Con)	Glu	160	147 (18)
	Glu+Arg	70	572 (12)
	Arg	120	605 (10)
ntrB(Con) *argR*::Tn*10*	Glu	160	15 (7)
	Glu+Arg	130	15 (7)
	Arg	300	101 (14)

[a] Cells were grown in glucose minimal medium (6) at 37°C. The following supplements were added as indicated: Glu, glutamate at 20 mM; Arg, arginine at 20 mM.

[b] AST was assayed by the procedure described by Vander Wauven and Stalon (28). The reaction mixture (2.0 ml) contained 100 mM Tris-HCl (pH 8.0), 0.3 mM succinyl-coenzyme A (CoA), and 10 mM L-arginine. The reaction was initiated by the addition of arginine at 37°C, and the decrease in the succinyl-CoA concentration was monitored at an absorbance of 232 nm. Protein concentration was determined by the method of Bradford (2). Standard errors are indicated in parentheses.

a functional ArgR is essential for cAMP-CRP-dependent induction of the *ast* operon under carbon starvation. These results also indicate that the concentration of the functional ArgR-arginine complex in the wild-type parent is not a limiting factor under conditions of carbon starvation.

Final conclusions. Computer analysis of the upstream region flanking the *ast* operon of *E. coli* (3, 23) led to the identification of two potential NtrC sites (also called NRI) that were presumed to function in nitrogen control of the operon. The results presented here identify two NtrC sites at the corresponding locations in the control region for the *ast* operon of *S. typhimurium* (Fig. 1). DNase I footprinting confirms that the two identified sites are in the center of a 55-bp region protected by NtrC (Fig. 3). The higher level of AST in the constitutive derivative, *ntrB*(Con), supports the conclusion that NtrC mediates nitrogen control of the *ast* operon in *S. typhimurium*.

TABLE 2. Effects of carbon starvation on expression of AST in wild-type *S. typhimurium* and its *crp*::Tn*10* and *argR*::Tn*10* derivatives

Genotype[c]	Supplement[a]	Sp act (nmol/mg/min)[b]
Wild type	0.2% Glucose	<0.1
	0.2% Glucose+Arg	<0.1
	0.03% Glucose	53 (4)
	0.03% Glucose+Arg	68 (2)
crp::Tn*10*	0.03% Glucose	<0.1
	0.03% Glucose+Arg	<0.1
argR::Tn*10*	0.03% Glucose	<0.1
	0.03% Glucose+Arg	<0.1

[a] Cells were grown in minimal medium (6) containing 20 mM $(NH_4)_2SO_4$ at 37°C. The final concentration of glucose in the culture was either 0.2% for carbon excess or 0.03% for carbon starvation. Cultures supplemented with 0.03% glucose ceased growth at an optical density of 0.5 at 600 nm and were harvested 2 h after glucose depletion. Arginine (Arg) was added at 20 mM. During logarithmic growth, the doubling time for the wild type and its *argR* derivative was 50 min whereas the doubling time for the *crp* derivative was 75 min.

[b] See footnote *b* of Table 1.

[c] The *crp*::Tn*10* locus from strain PP1037 (Salmonella Genetic Stock Center) was introduced into the wild type by phage P22 transduction. The *argR*::Tn*10* derivative was previously characterized (15).

The results presented here also clearly establish that inactivation of ArgR abolishes arginine induction of the *ast* operon in *S. typhimurium* under conditions of nitrogen limitation (Table 1). Gel retardation experiments showed that ArgR binds specifically to a DNA fragment carrying the region downstream of the NtrC binding sites. The observed affinity is similar to that previously determined for binding of ArgR to the arginine-repressible *car* operator of *S. typhimurium* (14). DNase I footprinting showed that ArgR protects a 90-bp fragment carrying two of the identified ArgR sites and that this protection is extended further downstream to a third site at higher ArgR concentrations.

The 3′ end of the proximal NtrC site is about 200 bp upstream of the putative σ^{54} promoter. Studies with the *glnA* promoter of *S. typhimurium* have shown that NtrC bound at the enhancer, located between −108 and −140, interacts directly with σ^{54} holoenzyme by means of DNA loop formation (25, 30). Our hypothesis is that in the case of the *ast* promoter, it is necessary that one or more ArgR molecules bind to the region between NtrC sites and the putative σ^{54} promoter in order to bring NtrC into proximity with RNA polymerase. The action of ArgR could occur through DNA bending or wrapping around the ArgR molecule. Studies with ArgR of *E. coli* (26, 29) and *S. typhimurium* (14) indicate that the binding of ArgR requires L-arginine and that a single hexamer binds through contacts with one face of the DNA helix in both the minor and major grooves. Crystallographic studies have shown that the hexameric form consists of two trimers and is greatly stabilized upon binding of six L-arginine molecules at the trimer-trimer interface (27). Accordingly, an increase in the L-arginine pool would increase the proportion of active ArgR with specific DNA binding activity, resulting in activation of the catabolic *ast* operon by NtrC.

In addition to arginine induction and nitrogen control, expression of the *ast* operon is also subject to carbon catabolite repression (1, 3). The AST pathway is induced under carbon starvation, and both ArgR and CRP are required for such induction (Table 2). Evidence for CRP binding to the site identified from the sequence (Fig. 1) was provided from the results of gel retardation experiments. While *S. typhimurium* and *E. coli* can utilize arginine as a sole nitrogen source but not as a sole carbon source (6), the AST pathway can also provide carbon skeletons that might become critical under conditions of carbon limitation. Induction by carbon starvation is most likely mediated at a promoter recognized by the σ^S subunit of enteric RNA polymerase. The participation of a σ^S promoter in expression of the *ast* operon in *E. coli* has recently been reported (3). The results presented here (Table 2) indicate that activation of this σ^S promoter by the cAMP-CRP complex also require a functional ArgR. The role of ArgR in this activation under conditions of carbon limitation could be similar to that proposed above for activation by NtrC under conditions of nitrogen limitation. The role proposed here for ArgR extends its functions beyond those previously recognized in enteric bacteria: namely, repression of genes of arginine biosynthesis (17) and resolution of ColE1 plasmid multimers (8).

Nucleotide sequence accession number. The nucleotide sequence determined in this study has been assigned GenBank accession no. AF108767.

We are indebted to Sydney Kustu (University of California at Berke-

ley) for the generous gift of purified NtrC and for helpful suggestions and stimulating discussions throughout this work. We thank P. C. Tai (Georgia State University) for the gift of purified CRP.

This work was supported in part by research grant GM47926 from the National Institute of General Medical Sciences.

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