

Interactions of the Antizyme AtoC with Regulatory Elements of the *Escherichia coli* *atoDAEB* Operon[∇]

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AtoC has a dual function as both an antizyme, the posttranslational inhibitor of polyamine biosynthetic enzymes, and the transcriptional regulator of genes involved in short-chain fatty acid catabolism (the *atoDAEB* operon). We have previously shown that AtoC is the response regulator of the AtoS-AtoC two-component signal transduction system that activates *atoDAEB* when *Escherichia coli* is exposed to acetoacetate. Here, we show that the same *cis* elements control both promoter inducibility and AtoC binding. Chromatin immunoprecipitation experiments confirmed the acetoacetate-inducible binding of AtoC to the predicted DNA region *in vivo*. DNase I protection footprinting analysis revealed that AtoC binds two 20-bp stretches, constituting an inverted palindrome, that are located at –146 to –107 relative to the transcription initiation site. Analyses of promoter mutants obtained by *in vitro* chemical mutagenesis of the *atoDAEB* promoter verified both the importance of AtoC binding for the inducibility of the promoter by acetoacetate and the σ^{54} dependence of *atoDAEB* expression. The integration host factor was also identified as a critical component of the AtoC-mediated induction of *atoDAEB*.

Escherichia coli utilizes a variety of carbon-containing compounds, including fatty acids, which are degraded by β -oxidation into acetyl coenzyme A (acetyl-CoA) (3). The utilization of long-chain (C₁₂ to C₁₈) and medium-chain (C₇ to C₁₁) fatty acids requires the actions of several proteins encoded by the *fad* regulon genes, whose products are involved in the transport, activation, and β -oxidation of fatty acids (reference 3 and references therein). The use of short-chain (C₄ to C₆) fatty acids also requires a membrane transporter (AtoE) and two catabolic enzymes, i.e., the acetyl-CoA–acetoacetate-CoA transferase (AtoD–AtoA) and the 3-ketoacyl-CoA thiolase II (AtoB) (12, 13). These proteins are encoded by the *atoDAEB* operon, which is highly inducible by acetoacetate but not by other saturated short-chain fatty acids, such as butyrate and valerate, whose catabolism also requires enzymes encoded by the *atoDAEB* operon genes (13, 25). Genetic analyses identified AtoC, a 48-kDa protein encoded by *atoC*, as the positive gene regulatory factor required for the inducible expression of the *atoDAEB* operon (13). Subsequent work identified the antizyme (Az) AtoC as the response regulator (RR) of a two-component system (TCS) that consists of AtoC and the sensor histidine kinase (HK) AtoS (15, 16).

Az was first described by Fong et al. and Heller et al. as a polyamine-inducible noncompetitive, proteinaceous inhibitor of ornithine decarboxylase, the key enzyme in polyamine biosynthesis (6, 10). In an attempt to identify the *E. coli* Az gene, we previously isolated and sequenced a 6.4-kb genomic DNA fragment encompassing part of the *atoDAEB* operon and the

genes of a putative HK and an RR, which proved to encode AtoS and AtoC, respectively (2). The identified *E. coli* Az gene showed significant sequence homology to genes for RRs of the NtrC–NifA family of the σ^{54} RNA polymerase transcriptional activators (24, 35, 40), and its genetic locus coincided with *atoC* (2). A gene that showed significant sequence similarities to genes for sensor HKs (24, 35) and localized just upstream of *atoC* was assigned the name *atoS* since it was presumed to encode the sensor HK that modulates AtoC activity (2). Recently, we presented both biochemical evidence on the phosphorylation of AtoC by AtoS and functional data on the positive regulation of the *atoDAEB* operon by the AtoS–AtoC TCS upon acetoacetate induction (16). Using AtoS-enriched membrane preparations as a kinase source and wild-type and mutant forms of AtoC as substrates in the phosphorylation reactions, we showed that mutant AtoC that lacks the conserved aspartic acid-55 (D55) due to a D55G substitution can be phosphorylated. Histidine-73 (H73) appears to be a second AtoC phosphorylation site since it is located within a characteristic “H-box” and since its alteration in the D55G/H73L double AtoC mutant leads to almost complete inhibition of AtoC phosphorylation *in vitro* (16).

Transcriptome and phenotypic microarray analyses (21, 45), however, have indicated that the role of *atoSC* may not be limited to the regulation of *atoDAEB* expression. Indeed, *E. coli* mutants with the *atoSC* locus deleted display altered gene expression (11 up-regulated and 32 down-regulated genes) (21), as well as reduced motility, sodium chloride sensitivity, and increased susceptibility to membrane-acting agents and an aminoglycoside antibiotic (21, 45). We have also demonstrated previously that *atoSC* can positively modulate the biosynthesis of poly-(R)-3-hydroxybutyrate, a biopolymer with many physiological roles in *E. coli* (38).

The aim of this work was to further advance our understand-

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TABLE 1. Bacterial strains, plasmids, and oligonucleotide primers used in this work

Strain, plasmid, or oligonucleotide	Characteristics or description	Source and/or reference
<i>E. coli</i> strains		
Top10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697	Invitrogen
BL21(DE3)	<i>galU galK rpsL</i> (Str ^r) <i>endA1 nupG</i> λ ⁻	Novagen
N99	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3)	M. Gottesman (9)
N7193	Wild type; λ ⁻ F ⁻ <i>sup⁰ galK2</i>	M. Gottesman (9)
BW25113	N99 Δ <i>hip-3</i> ::CAT	H. Aiba (21)
BW28878	<i>lacI^r rrrB3</i> Δ <i>lacZ4787</i> <i>hsdR514</i> Δ(<i>araBAD</i>)567 Δ(<i>rhaBAD</i>)568 <i>rph-1</i> BW25113 Δ(<i>atoSC</i>)569	H. Aiba (21)
Plasmids		
pZErO 2.1	Cloning vector	Invitrogen
pZero-patoD1	<i>atoD1p</i> PCR product (-206 to +73 of <i>atoDAEB</i>) cloned into the EcoRV site of pZErO 2.1	This work
pZero-patoD2	<i>atoD2p</i> PCR product (-120 to +73 of <i>atoDAEB</i>) cloned into the EcoRV site of pZErO 2.1	This work
pZero-patoD3	<i>atoD3p</i> PCR product (-47 to +73 of <i>atoDAEB</i>) cloned into the EcoRV site of pZErO 2.1	This work
pMLB1034	Medium-copy-number vector containing a promoterless <i>lacZ</i>	34
pMLB-atoD1	<i>atoD1p</i> (EcoRI-BamHI fragment from pZero-patoD1) cloned into pMLB1034	This work
pMLB-atoD2	<i>atoD2p</i> (EcoRI-BamHI fragment from pZero-patoD2) cloned into pMLB1034	This work
pMLB-atoD3	<i>atoD3p</i> (EcoRI-BamHI fragment from pZero-patoD3) cloned into pMLB1034	This work
pCPG3	<i>resC</i> regulatory sequences (-656 to +570) cloned into pMLB1034	This work
pCPG4	<i>atoS</i> regulatory sequences (-729 to +189) cloned into pMLB1034	This work
pCPG5	<i>atoDAEB</i> regulatory sequences (-419 to +1186) cloned into pMLB1034	This work
pCPG6	<i>atoC</i> regulatory sequences (-1150 to +187) cloned into pMLB1034	This work
Oligonucleotides		
U1patoD	5'-TCGGAATTCATTGATGTATAAACTCCAGGAA-3'	This work
U2patoD	5'-AGGGAAATCAATTTCTGCATAGCCGCTCAT-3'	This work
U3patoD	5'-TACGAATTCACATAAATCCAAATAATCTCATT-3'	This work
LpatoD	5'-AAGGGATCCGCTGGCGCTTGTAAATGTTCAT-3'	This work

ing of the role of the AtoS-AtoC TCS in gene regulation by (i) characterizing the *cis* elements within the *atoDAEB* promoter regulatory region that are responsible for the induction of the operon by acetoacetate, (ii) mapping and identifying the AtoC binding site(s) within this region and, (iii) identifying additional factors that affect AtoC function.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The genotypes of the *E. coli* strains used in this work are described in Table 1. *E. coli* cells were grown at 37°C either in modified M9 mineral medium supplemented with 0.1 mM CaCl₂, 1 mM MgSO₄, 0.4% (wt/vol) glucose, 1 μg of thiamine/ml, 80 μg of proline/ml, and 1.7 μM FeSO₄ (16) or in Luria-Bertani broth (28).

Plasmid constructions. The *atoD1*, *atoD2*, and *atoD3* regions upstream of the *atoDAEB* operon, extending, respectively, from -206, -120, and -47 to +73 relative to the predicted transcriptional start site, were PCR amplified from plasmid pUC-Az (2) by using different forward primers (U1patoD, U2patoD, and U3patoD) and a common reverse primer (LpatoD) (Table 1). The PCR products were cloned into the EcoRV site of pZErO 2.1 (Invitrogen) to construct plasmids pZero-patoD1, pZero-patoD2, and pZero-patoD3, respectively, and then subcloned into the medium-copy-number plasmid vector pMLB1034 (EcoRI and BamHI sites) containing a promoterless *lacZ* gene (34) to yield plasmids pMLB-atoD1, pMLB-atoD2, and pMLB-atoD3 (Table 1).

The 930-bp EcoRI-HincII fragment from pCPC-J (2) containing the *atoS* promoter was introduced into EcoRI-SmaI-digested pMLB1034 to produce pCPG4 (*atoS lacZ*). The 1.2-kb EcoRI-HincII fragment from pCPC-Az (2) containing the *atoDAEB* promoter and sequences coding for AtoD and the N-terminal 21 amino acids of AtoA was cloned into EcoRI-SmaI-cut pMLB1034 to generate pCPG5 (*atoA lacZ*). The 1.3-kb EcoRV fragment from pUC-Az containing the *atoC* promoter was introduced into SmaI-cut pMLB1034 to yield pCPG6 (*atoC lacZ*). The 1.2-kb BamHI-EcoRI fragment from pUC-Az containing the *resC* (19) promoter was cloned into EcoRI-BamHI-cut pMLB1034 to generate pCPG3 (*resC lacZ*).

β-Galactosidase assays. β-Galactosidase assays were performed using permeabilized *E. coli* cells carrying the appropriate plasmids, as described previously

(16, 20). The cells had been grown in modified M9 mineral medium (28) supplemented with 0.1 mM CaCl₂, 1 mM MgSO₄, 0.4% (wt/vol) glucose, 1 μg of thiamine/ml, 80 μg of proline/ml, and 1.7 μM FeSO₄ in the absence or presence of 10 mM acetoacetate.

AtoC purification and electrophoretic mobility shift assays (EMSAs) of DNA binding. The recombinant AtoC (rAtoC) was purified (16) and dialyzed against a mixture of 10 mM HCl, 50% (vol/vol) glycerol, and 5 mM β-mercaptoethanol. Following the determination of the rAtoC concentration and an analysis of its purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the protein was stored in aliquots at -80°C.

The DNA binding activity of the purified rAtoC (16) was determined by nonradioactive EMSAs using biotin-labeled probes. The probes were prepared by PCR amplification using pZero-patoD1 DNA as the template and primer pairs consisting of a biotinylated M13 universal primer and either the U1patoD, U2patoD, or U3patoD primer (see above). The DNA binding reactions took place in a 20-μl final volume for 30 min at 4°C by incubating purified rAtoC (0.008 to 0.7 μM) in a mixture of 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5% (vol/vol) glycerol, 1 mM EDTA, 4 mM dithiothreitol, 0.2 mg of bovine serum albumin/ml, and 10 ng of the probe (approximately 3 nM) in the presence of 2 μg of a nonspecific DNA competitor (sonicated salmon sperm DNA). In competition assays, the mixtures were incubated with the appropriate concentration of a competitor prior to the addition of the probe to the reaction mixture. Supershift analyses were performed as previously described (23) using either a specific monoclonal antibody (MAb) such as HIS-1 (Sigma) that recognizes the rAtoC polyhistidine tag or the nonspecific 58S MAb that recognizes an unrelated protein, i.e., the herpes simplex virus type 1 transcriptional regulator ICP4 (31).

Following electrophoretic separation on 4% (wt/vol) polyacrylamide gels with 0.25× Tris-borate-EDTA buffer, the DNA-protein complexes were electroblotted onto 0.45-μm-pore-size Biotodyne B membranes (PALL) for 1 h at 30 V in 0.5× Tris-borate-EDTA. The membranes were cross-linked with UV light (0.120 J) and blocked for 5 min with a solution of 5% (wt/vol) sodium dodecyl sulfate, 125 mM NaCl, 17 mM Na₂HPO₄, and 8 mM NaH₂PO₄, pH 7.2 (0.1 ml/cm²), and then the blots were developed using the Phototope-Star detection kit according to the instructions of the manufacturer (NEB).

DNase I footprinting. PCR amplification was performed to prepare the DNA probes used in the DNase I footprinting analysis of the *atoDAEB* promoter, along with one nonradioactive and one 5'-end-³²P-labeled primer (U1patoD and

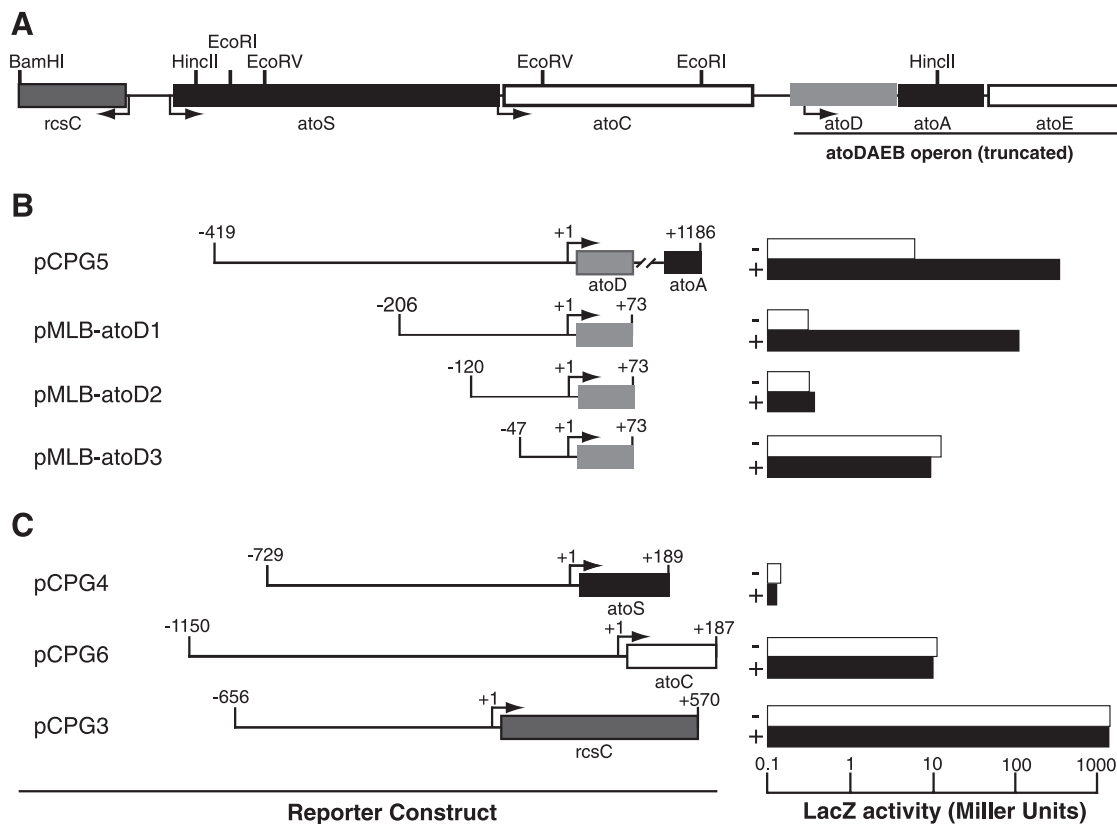


FIG. 1. Mapping of the acetoacetate-responsive *cis* element within the *atoDAEB* promoter. (A) Organization of the *E. coli* genomic sequences originally cloned into pUC-Az (2). The restriction enzyme sites used in the generation of reporter constructs are indicated. (B and C) *E. coli* Top10 cells carrying the indicated reporter constructs were grown in modified M9 mineral medium (28) in the absence (–; empty bars) or presence (+; filled bars) of 10 mM acetoacetate, and β -galactosidase (LacZ) activity in permeabilized cells was measured as described previously (20). (B) Susceptibility of reporter constructs carrying various regions of the *atoDAEB* promoter fused to the *lacZ* reporter gene to induction by acetoacetate. (C) Effects of acetoacetate on the activity of the *atoS*, *atoC*, and *rscC* promoters.

LpatoD) in each reaction. Labeled probes were purified using a Nucleospin extract kit (Macherey-Nagel) and mixed with various amounts of rAtoC (0.07 to 3.5 μ M) before the DNase I protection footprinting analysis (22).

ChIP assays. Chromatin immunoprecipitation (ChIP) from *atoSC*⁺ or Δ *atoSC* bacteria carrying either the pMLB-atoD1 or the pMLB-atoD2 plasmid was performed as previously described (8). Briefly, in vivo cross-linking of the bacterial nucleoproteins took place by the addition of formaldehyde (final concentration, 1% [vol/vol]) directly to the *E. coli* cultures growing in modified M9 mineral medium (28). After 20 min, glycine (300 mM) was added to quench the cross-linking reactions and the cells were collected by centrifugation at 4°C and washed twice with ice-cold Tris-buffered saline. Cell lyses, chromatin preparations, and ChIPs were performed as previously described (8) using an anti-AtoC rabbit polyclonal antibody (2). The presence of the plasmid-borne *atoDAEB* promoter sequences in the chromatin immunoprecipitates was detected by *atoDAEB* promoter-*lacZ* fusion-specific PCR amplification using a primer pair consisting of U2patoD and a universal M13 forward primer. Following 27 cycles of amplification, the PCR products were analyzed on 3% (wt/vol) low-melting-point agarose gels (Nusieve) and visualized by ethidium bromide staining.

Hydroxylamine mutagenesis. Plasmid pZero-patoD1 (described above), carrying the *atoDAEB* promoter region from –206 to +73, was mutagenized in vitro with hydroxylamine (33). The mutagenized promoter sequences were excised by EcoRI-BamHI digestion and cloned into pMLB1034 (34). Competent *E. coli* Top10 cells were transformed with the ligation products, and the cells were plated onto M9 mineral medium-agar plates containing 10 mM acetoacetate and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). Plasmid DNA was isolated from white or pale blue colonies, and the *atoDAEB* promoter region was sequenced using standard methodology.

RESULTS

Identification of the acetoacetate-responsive *cis* element within the *atoDAEB* promoter regulatory region. To define the acetoacetate-responsive *cis* element of *atoDAEB*, we constructed a series of galactosidase reporter translational fusions with *atoDAEB* subregions and monitored reporter expression in the absence or presence of the inducer. The reporter constructs pCPG5 and pMLB-atoD1 (Table 1), carrying the –419-to-+1186 and –206-to-+73 regions of *atoDAEB*, respectively (Table 1), were inducible by acetoacetate (Fig. 1B). However, further deletion of 86 or 159 bp from the 5' end rendered the resulting constructs, pMLB-atoD2 and pMLB-atoD3 (Table 1), unresponsive to acetoacetate induction (Fig. 1B). Therefore, the acetoacetate-responsive element maps, at least partly, within the 86-bp region from –206 to –120 of the *atoDAEB* promoter (Fig. 1B).

To determine whether acetoacetate affects *atoDAEB* indirectly by altering the expression of the genes encoding its regulatory AtoS-AtoC TCS (15, 16), we also generated *lacZ* reporter fusion constructs with the –729-to-+189 region of *atoS* (pCPG4) or the –1150-to-+187 region of *atoC* (pCPG6). The expression of the *lacZ* reporter from these constructs was

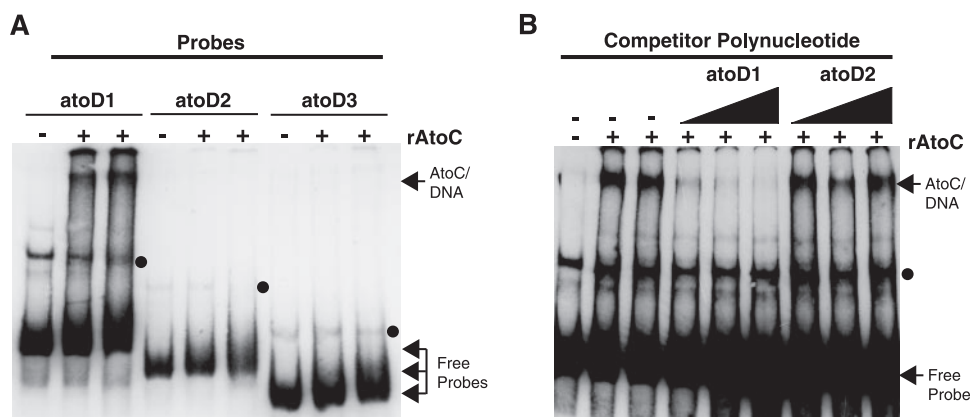


FIG. 2. Mapping of the AtoC binding site(s) within the *atoDAEB* promoter. (A) The binding of AtoC (35 nM) to *atoDAEB* promoter regions *atoD1* (−206 to +73), *atoD2* (−120 to +73), and *atoD3* (−47 to +73) was analyzed by nonradioactive EMSAs. (B) The DNA binding reactions were performed in the presence of 10 ng of the *atoD1* probe (approximately 3 nM) and increasing amounts (5-, 10-, and 20-fold molar excesses) of either unlabeled specific (*atoD1*) or nonspecific (*atoD2*) competitors (see Materials and Methods) and analyzed by EMSAs. The AtoC-DNA complexes and the free probes are indicated by arrows, whereas the protein-independent bands are indicated by dots. +, present; −, absent.

unaffected by the presence of acetoacetate (Fig. 1C). The expression of *rcsC* (36), a neighboring gene that is transcribed in the opposite direction (Fig. 1A) and that was also monitored as a specificity control, was not affected by acetoacetate (Fig. 1C).

In vitro analysis of AtoC binding to *atoDAEB* regulatory sequences. To locate the AtoC binding sites within the *atoDAEB* promoter, we generated three overlapping DNA fragments that shared the same 3' end (at +73 relative to the putative transcription start site) but differed at the 5' end (Fig. 1) and tested them for the ability to bind AtoC. The affinity-purified rAtoC (16) used in these experiments was in its unphosphorylated state, since we were unable to phosphorylate it to a significant extent in vitro either with its cognate AtoS HK or with low-molecular-weight phosphodonors like carbamoyl phosphate, phosphoramidate, and acetyl phosphate (data not shown). Unphosphorylated RRs retain their ability to bind specifically their DNA target sequences, although phosphorylation enhances their affinity for DNA (27, 32, 43).

EMSA analysis revealed that while AtoC binds *atoD1* (−206 to +73), it fails to bind *atoD2* (−120 to +73) and *atoD3* (−47 to +73) (Fig. 2A), suggesting that the sequences necessary for AtoC binding lie within the region from −206 to −120 relative to the predicted *atoDAEB* transcriptional start site. These data indicate that the same *cis* element, i.e., the region from −206 to −120, is involved in both AtoC binding and the acetoacetate responsiveness of *atoDAEB* and support the notion that AtoC is the transcription factor involved in the induction of *atoDAEB* by acetoacetate.

The specificity of the above-described DNA-AtoC interactions was verified by competition experiments to evaluate the association of AtoC with biotinylated *atoD1* in the presence of increasing concentrations of an unlabeled specific (*atoD1*) or nonspecific (*atoD2*) DNA competitor. The addition of increasing amounts of the specific *atoD1* competitor greatly decreased the abundance of AtoC-DNA complexes, whereas similar concentrations of the nonspecific *atoD2* competitor produced no visible effect (Fig. 2B). Finally, to further verify the presence of AtoC in the *atoD1* EMSA complexes, we included in the reaction mixtures either a MAb that specifically recognizes poly-

histidine stretches, such as the His₁₀ tag of rAtoC, or a non-specific MAb (58S) that recognizes an unrelated viral protein (31). While the nonspecific 58S MAb had no effect on the AtoC-DNA complexes, the specific anti-polyhistidine MAb clearly decreased the abundance of these complexes (data not shown). These data further demonstrate the presence of rAtoC in the EMSA complexes with the *atoD1* probe.

Characterization of the AtoC binding sites by DNase I footprinting. Having shown that the region from −206 to −120 of *atoDAEB* is involved in both physical interactions with AtoC and promoter inducibility by acetoacetate, we performed DNase I protection footprinting analysis to identify the exact contacts of AtoC with promoter sequences. Probes labeled in either the upper or lower strand were subjected to DNase I nicking in the presence of increasing concentrations of AtoC (Fig. 3A). The analysis of the upper-strand-labeled promoter indicated that AtoC protects mainly the region from −146 to −110 from DNase I digestion. This region contains an inverted palindrome of the consensus sequence GCTATGCAGAAW TTGCRCA in regions −146 to −127 and −126 to −107, between which the two halves are separated by 1 bp (Fig. 3B). The protection of these sites was verified by footprinting analysis of the lower strand (Fig. 3A). The locations of these AtoC binding sites are in good agreement with the relative positions of enhancer-like elements (ELEs) present in other σ^{54} -dependent promoters (references 26 and 41 and references therein). AtoC binding induces significant DNase I hypersensitivity (Fig. 3), especially at positions −101, −109, and −128, indicating that significant structural DNA alterations take place upon AtoC binding. It is possible that such structural changes facilitate the interaction of AtoC with the σ^{54} RNA polymerase holoenzyme that is bound to the region from −24 to −12 (Fig. 3B).

ChIP analysis of in vivo AtoC binding to *atoDAEB* regulatory sequences. The above-described in vitro determination of the AtoC binding site within the *atoDAEB* regulatory sequences was also investigated in vivo by performing ChIP experiments (8) using *atoSC*⁺ (BW25113) or Δ *atoSC* (BW28878) bacteria carrying plasmids that either contained the AtoC

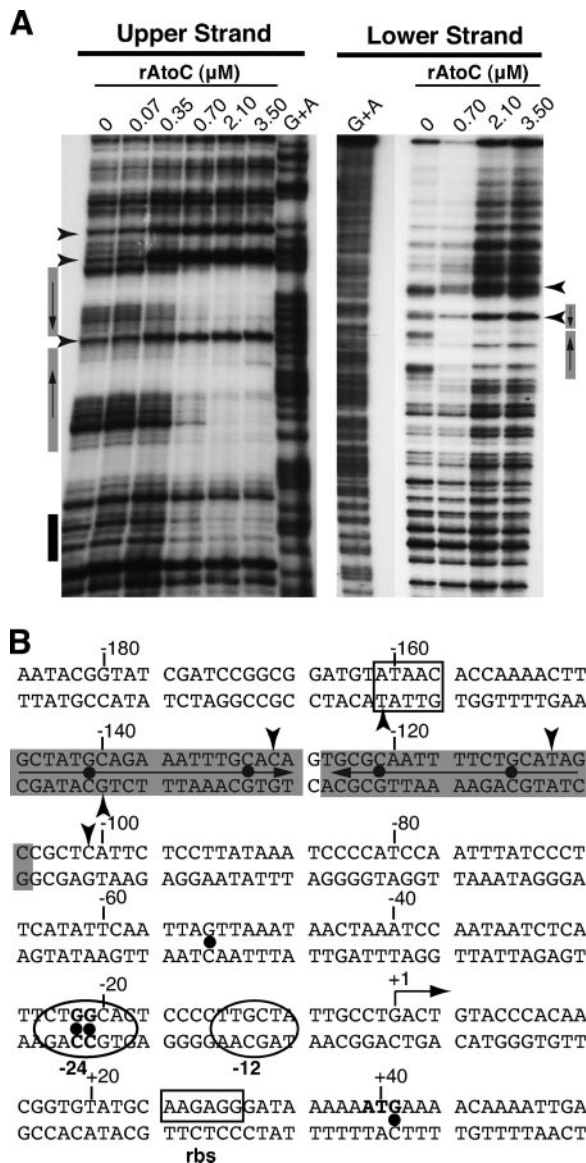


FIG. 3. DNase I footprinting analysis of the AtoC binding sites within the *atoDAEB* promoter regulatory region. (A) DNase I footprinting was performed with increasing concentrations of rAtoC and promoter fragments labeled in the coding (upper) or noncoding (lower) strand. Boxes indicate the protected areas, and arrowheads indicate the hypersensitivity positions. The gray boxes indicate the inverted palindromes protected by AtoC (see the text). (B) The *atoDAEB* sequence (−186 to +54) and its regulatory elements are shown. Note that the transcription initiation site shown in this figure is predicted and has not been validated experimentally. Shaded sequences represent the AtoC-protected areas, and arrowheads indicate AtoC-induced hypersensitivity. The positions of some predicted functional elements, such as the promoter region from −24 to −12 (in ovals), the putative ribosome binding site (boxed), and the initiator ATG (bold), are indicated. The positions of the hydroxylamine-induced mutations that render the promoter inactive and/or unresponsive to acetoacetate (Fig. 5 and 6) are marked by dots.

binding site (pMLB-atoD1) or lacked it (pMLB-atoD2). Figure 4 shows that AtoC binds *in vivo* to the plasmid-borne *atoDAEB* sequence containing an AtoC binding site (*atoD1*, −206 to +73) whereas it fails to bind sequences lacking this

site (*atoD2*, −120 to +73). Furthermore, the abundance of the AtoC-DNA complexes increased significantly in cells growing in the presence of acetoacetate. The specificity of these interactions was underscored by the lack of PCR products in the absence of antibody or in immunoprecipitates from the Δ *atoSC* *E. coli* strain BW28878 that lacks AtoC (Fig. 4). Thus, AtoC displayed the same sequence requirements for DNA binding both *in vitro* and *in vivo*, and the abundance of the AtoC-DNA complexes increased upon the activation of the AtoS-AtoC TCS by acetoacetate.

Characterization of the *atoDAEB* promoter regulatory elements by chemical mutagenesis. To further characterize the functional *cis* elements within the *atoDAEB* promoter, we used *in vitro* hydroxylamine mutagenesis (33) and selected for severely reduced promoter activity in the presence of acetoacetate. This relatively unbiased approach was expected to yield two major types of mutations: (i) those that alter the core promoter elements, i.e., the sequence from −24 to −12 making contact with the σ^{54} holoenzyme of RNA polymerase, and (ii) those that render the promoter refractory to acetoacetate induction, e.g., by altering the AtoC binding site or the binding site of an AtoC cofactor protein. Indeed, sequence analysis of 24 mutants that displayed no, or greatly reduced, promoter activity in the presence of acetoacetate indicated that the majority of the mutants (16 out of 24) carried single mutations whereas the rest carried two or more mutations.

The majority of the single mutants (9 of 16) carried G-to-A transitions at positions −21 and −22 relative to the predicted transcription start site (Fig. 3B). Upon acetoacetate induction, the expression levels of the *lacZ* reporter in these mutants were severely reduced compared with that of the wild-type *atoDAEB* promoter regulatory sequence (Fig. 5). Four mutations that mapped within the AtoC binding site (−111, −121, −130, and −140) (Fig. 3B) resulted in significant decreases ($\geq 80\%$) in the induced *atoDAEB* promoter activity (Fig. 5). Interestingly enough, one mutation that mapped at position −53, i.e., between the AtoC and the polymerase binding sequences (Fig. 3B), produced a more pronounced phenotype than any of the AtoC binding site mutations (Fig. 5). This result may be due either to a mutation-induced gross alteration in the DNA structure that also affects the AtoC binding or to the destruction of the binding site of another protein participating in the induction of the *atoDAEB* operon.

To determine whether the mutation at position −53 affects AtoC binding, as well as to confirm that the AtoC binding site mutations do affect the binding of AtoC, we tested the ability of probes carrying wild-type or mutant sequences to bind AtoC *in vitro*. The results of this experiment indicated that AtoC binding to probes carrying a single mutation within the AtoC binding site (at position −140, −121, or −111) was greatly reduced, whereas AtoC binding to a probe carrying the −53 mutation was indistinguishable from AtoC binding to the wild-type probe (Fig. 6). Therefore, the phenotype corresponding to the mutation at position −53 was not due to reduced AtoC binding.

The integration host factor (IHF) is required for the induction of the *atoDAEB* operon. The *atoDAEB* promoter contains two sequence stretches (−62 to −50 and −37 to −25) that bear homology to IHF binding sites (data not shown). The presence of architectural factors, like IHF, is often required for

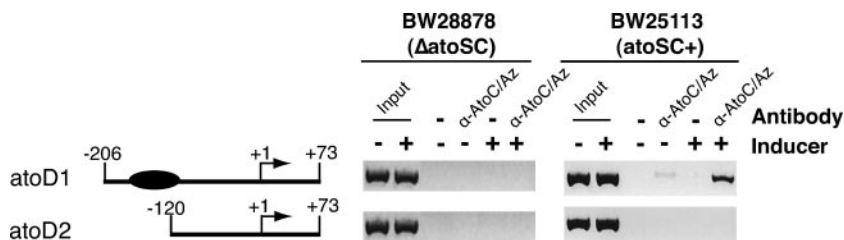


FIG. 4. ChIP analysis of AtoC binding to *atoDAEB* promoter fragments in vivo. The binding of AtoC to plasmid-borne *atoDAEB* promoter fragments that either carry (*atoD1*) or lack (*atoD2*) the predicted binding site of AtoC (filled oval) in the presence (+) and absence (-) of acetoacetate (inducer) was determined. The *atoSC*⁺ strain BW25113 and its Δ *atoSC* isogenic counterpart BW28878, carrying either the plasmid pMLB-*atoD1* or pMLB-*atoD2* (Table 1), were used in this experiment. Immunoprecipitations were performed in the absence (-) or presence of anti-AtoC (α -AtoC/Az) antibodies, and they were followed by PCR amplification of the immunoprecipitated DNA fragments by using one *atoDAEB*-specific (U2patoD [Table 1]) and one plasmid-specific (universal M13 forward) primer. Chromatin input controls are indicated.

the optimal contact of the ELE-bound NtrC-type transcription factor with the promoter-bound σ^{54} RNA polymerase (11, 26, 29, 41). Since the -53 mutation lies within one putative IHF binding site, we tested whether IHF is involved in the acetoacetate-induced expression of *atoDAEB*. Plasmids carrying the *lacZ* reporter under the control of either the wild-type promoter (*atoD1*) or a promoter with one mutation (-53 or -140) were introduced into both the wild-type *E. coli* strain N99 and its isogenic counterpart, N7193 (Table 1), which has an IHF null phenotype due to an insertion in the *hip* (*himD*) gene (9). *LacZ* activities in cells growing in the absence or presence of acetoacetate were monitored. The results of this experiment indicated that (i) the induction of *atoDAEB* in the IHF null N7193 strain was greatly reduced compared to that in wild-type N99 cells and (ii) in the IHF null cells, the wild-type *atoDAEB* promoter could not be differentiated from a mutant carrying an AtoC binding site mutation on the basis of acetoacetate inducibility (Fig. 7). This finding is in contrast with results for induced wild-type N99 cells, in which the *LacZ* reporter activity corresponding to the promoter with the -140 AtoC binding site mutation was more than 70% lower than

that corresponding to the wild-type promoter (Fig. 7). These data indicated that the presence of IHF is required for the AtoC-mediated induction of the *atoDAEB* operon.

DISCUSSION

AtoC possesses diverse biological roles both as a posttranslational inhibitor of polyamine biosynthetic enzymes and as a transcriptional regulator of *atoDAEB* expression. The aim of the experiments reported here was to identify regulatory elements and factors that are involved in the transcriptional regulator function of AtoC.

The expression of the *atoDAEB* operon, whose gene products participate in the catabolism of short-chain fatty acids, is highly inducible by acetoacetate (13, 25). This induction requires AtoC (13), a transcription factor homologous to NtrC-NifA-type bacterial transcriptional regulators of the σ^{54} RNA polymerase holoenzyme (15, 26). These transactivators are usually part of two-component signal transduction systems that typically consist of a sensor HK and an RR, the latter of which is often a transcription factor (24, 35, 40). Upon sensing envi-

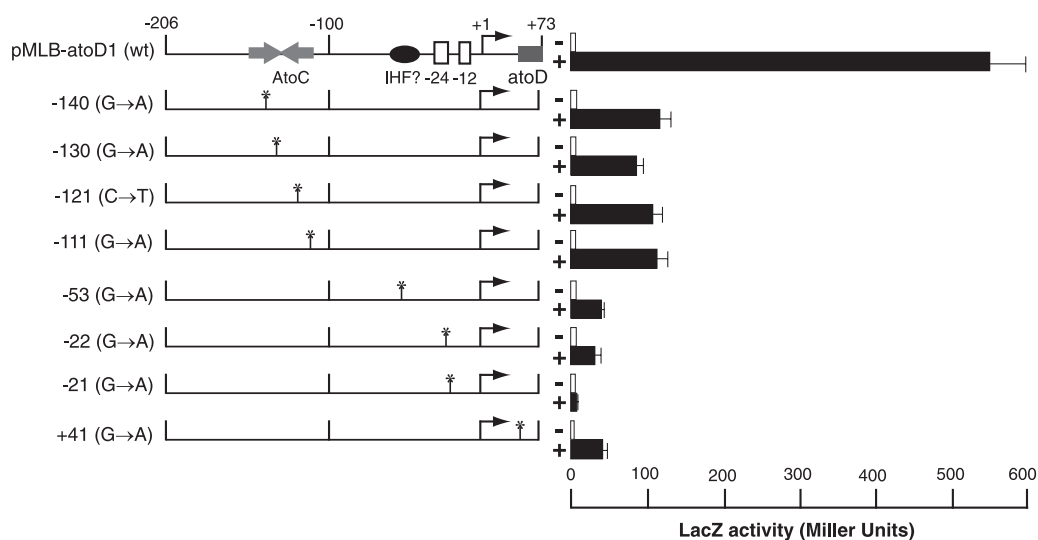


FIG. 5. Acetoacetate inducibility of *atoDAEB* promoter mutants obtained by chemical mutagenesis. *E. coli* Top10 cells carrying wild-type or mutant *atoDAEB* promoters fused to the *lacZ* reporter gene were grown in modified M9 mineral medium (28) in the absence (-; empty bars) or presence (+; filled bars) of 10 mM acetoacetate, and β -galactosidase (*LacZ*) activity in permeabilized cells was measured as described previously (20). The position of each mutation is indicated on the left. wt, wild type.

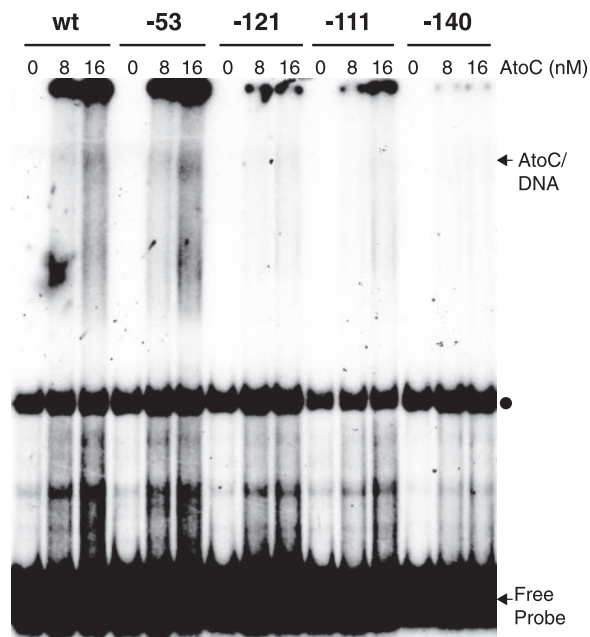


FIG. 6. In vitro binding of AtoC to wild-type and mutant *atoDAEB* promoter fragments. The binding of purified rAtoC (8 and 16 nM) to wild-type or mutant *atoDAEB* promoter (−206-to-+73) probes was analyzed by EMSAs. The mutations are identified at the top of each lane. AtoC-DNA complexes and free probes are indicated by arrows, whereas the positions of protein-independent bands are indicated by dots. wt, wild type.

ronmental or other signals, these HKs dimerize and autophosphorylate by *trans*-phosphorylation on conserved histidine residues. The phosphorylated HKs then transfer their phosphoryl groups to cognate RRs, usually to a conserved aspartate residue, leading to the multimerization and activation of the RRs. The activated RRs subsequently trigger the expression of the target operon(s), thus mounting a cell response to the received stimuli (24, 35, 40). We have previously shown that AtoC phosphorylation by the HK AtoS is required for the induction of *atoDAEB* expression upon the exposure of *E. coli* to acetoacetate (15, 16). By performing a deletion analysis of the *atoDAEB* promoter regulatory region, it was found that acetoacetate-mediated transcriptional activation requires *cis* elements mapping within the region from −206 to −120. Moreover, we showed that this induction is not due to altered levels of the AtoS-AtoC TCS proteins, since acetoacetate does not augment the expression of their genes, i.e., *atoS* and *atoC*.

We and others have reported previously that the in vitro AtoC phosphorylation by its cognate AtoS HK is rather inefficient (references 16 and 42 and unpublished data). Additionally, low-molecular-weight phosphodonors (5, 17, 18) such as carbamoyl phosphate, phosphoramidate, and acetyl phosphate that have been shown to phosphorylate a number of RRs in vitro failed to phosphorylate AtoC as well as they phosphorylate the other RRs (data not shown). It is not clear yet whether this failure to obtain significant amounts of phosphorylated AtoC represents a lack of phosphorylation or rapid AtoC-P dephosphorylation due to either chemical instability or phosphatase activity. By necessity, therefore, we used unphosphorylated AtoC in the in vitro protein-DNA binding experiments

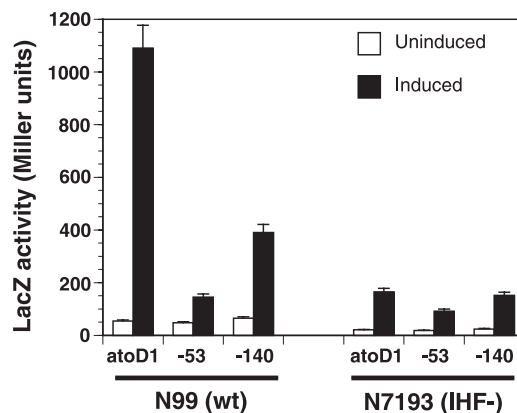


FIG. 7. Effect of the IHF on the acetoacetate inducibility of wild-type and mutant *atoDAEB* promoters. A pair of isogenic wild-type (N99 [wt]) or IHF-negative (N7193 [IHF⁻]) *E. coli* strains (Table 1) were used to monitor the activities of the wild-type *atoDAEB* promoter (*atoD1*) and mutant *atoDAEB* promoters (with a mutation at position −53 or −140) in the absence (empty bars) or presence (filled bars) of 10 mM acetoacetate. The promoters had been fused to the *lacZ* reporter gene, and the activity of β -galactosidase (LacZ) in permeabilized cells was measured as described previously (20).

performed in the course of this work. Although RRs normally require prior phosphorylation by an HK to bind their target sequences in vivo (reference 35 and references therein), in vitro experiments have shown that unphosphorylated RRs retain their sequence-specific DNA binding activities, albeit with lower affinity (27, 32, 43). The observation that active forms of RRs exist in unphosphorylated preparations of these proteins has led to the suggestion of the two-state equilibrium hypothesis, which postulates that phosphorylation alters the equilibrium between the active and inactive conformations of an RR (7, 39). Indeed, our in vivo data have indicated that overexpressed AtoC can activate its target *atoDAEB* promoter even when it is unable to phosphorylate due either to a lack of its cognate AtoS HK or to phosphorylation site mutations (16).

The in vitro DNA binding data presented here demonstrated that purified rAtoC binds specifically within the same sequences that are involved in the acetoacetate responsiveness of the *atoDAEB* operon, i.e., the region from −206 to −120. Note that the binding of AtoC to its target sequences is not enhanced when acetoacetate is included in the in vitro binding reactions (data not shown), which is consistent with the notion that acetoacetate acts by triggering the AtoS-AtoC TCS. The in vitro findings were verified by ChIP experiments, which demonstrated that the exposure of *E. coli* to acetoacetate induces the binding of AtoC to the *atoDAEB* regulatory sequences from −206 to −120 in vivo. Thus, AtoC appears to display the same DNA binding specificity both in vivo and in vitro.

The exact location of AtoC binding was examined further using DNase I footprinting analysis, which revealed that AtoC binds two 20-bp regions constituting an inverted palindrome that are located at −146 to −127 and −126 to −107 relative to the predicted *atoDAEB* transcription start site (Fig. 3). The sequence of each 20-bp AtoC binding site conforms to the consensus GCTATGCAGAAWTTGCRCA, and their locations are in good agreement with the relative positioning of

other ELEs present in σ^{54} -dependent promoters (references 26 and 41 and references therein). The importance of the presence of both copies of the AtoC binding site for the acetoacetate-dependent inducibility of *atoDAEB* was established from the results of chemical mutagenesis experiments. These results indicated that even single-base mutations within either of the two AtoC binding sites significantly reduced both the in vitro binding of AtoC to the mutant sites and the inducibility of the mutant promoters by acetoacetate. Thus, the integrity of both AtoC binding sites within the *atoDAEB* regulatory region is critical both for AtoC binding to DNA and for its effect on gene expression, possibly because this integrity is required for the cooperative DNA binding of this transcription factor (43).

We found another interesting mutation at -53 that reduced the inducibility of *atoDAEB* without affecting the binding of AtoC. This mutation lay within one of the two putative IHF binding sites that are located at -62 to -50 and -37 to -25 , between the AtoC and RNA polymerase binding sites. IHF has been shown to bind several promoters that are regulated by NtrC-type transcription factors, and its binding is believed to facilitate the optimal contact of the ELE-bound transcription factor with the promoter-bound σ^{54} RNA polymerase (11, 26, 29, 41). Indeed, IHF is required for *atoDAEB* induction, so that *E. coli* cells lacking functional IHF due to an insertion in the *hip* (*himD*) gene (9) are severely impaired with regard to the levels of *atoDAEB* promoter inducibility by acetoacetate.

The chemical mutagenesis data also provide experimental evidence of the transcription of the *atoDAEB* promoter by the σ^{54} RNA polymerase, a relationship that was suspected based on sequence similarities but lacked experimental verification thus far (26). This confirmation stems primarily from the finding that the majority of the analyzed mutations that impair the expression of *atoDAEB* are located in a conserved GG doublet (at positions -21 and -22) whose significance in σ^{54} holoenzyme complex formation was recognized quite early (reference 14 and references therein) and was verified recently following the compilation and analysis of a large number of σ^{54} -dependent bacterial promoter sequences (1).

Recent data posit the involvement of the AtoS-AtoC TCS in the expression of a number of *E. coli* genes other than those of the *atoDAEB* operon. Indeed, the deletion of the *E. coli* *atoSC* locus results in the up-regulation of 11 genes and the down-regulation of 32 genes (21), suggesting both positive and negative roles for the AtoS-AtoC TCS. While some of these effects may be indirect, it has been shown previously that RRs can be involved in both positive and negative gene regulation. For example, NtrC activates the σ^{54} -dependent *glnAp₂* promoter but represses the σ^{54} -dependent *glnAp₁* promoter in *E. coli* (30), while BvgA functions as both a transcriptional activator of multiple virulence factors and a repressor of genes required for motility and/or survival in several *Bordetella* species (4). This differential regulation may be due to a strategic placement of the binding sites and/or the concentration and the phosphorylation state of the RR. While BLAST analysis indicated that there are no palindromes consisting of two inverted AtoC binding sites other than the one at *atoDAEB* in the *E. coli* genome, it did show several regions with significant homology to the 20-bp AtoC binding site (data not shown). It is not clear whether any of these sites are recognized in vivo by either AtoC or an AtoC-containing complex or if they represent

targets of regulation. However, it is clear that AtoC is more than a regulator of the genes that participate in short-chain fatty acid catabolism since it affects a number of other physiological processes, such as flagellum biosynthesis and motility, the biosynthesis of the biopolymer poly-(R)-3-hydroxybutyrate, and processes that influence the sodium sensitivity but not the potassium sensitivity of *E. coli* cells or their susceptibility to membrane-acting agents and aminoglycoside antibiotics (21, 38, 45).

It remains to be elucidated why AtoC evolved both as a posttranslational regulator of polyamine biosynthetic enzymes and as a transcriptional factor for genes that encode proteins involved in other aspects of the *E. coli* physiology and metabolism. It is tempting to speculate that the reason relates to the diverse effects exerted by the polyamines on the expression of the *E. coli* genes sometimes described as the polyamine modulon (44), which includes genes involved in flagellar synthesis (44), and the expression of promoters transcribed by the σ^{54} RNA polymerase (37).

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