

# Regulation of Expression of the Divergent *ulaG* and *ulaABCDEF* Operons Involved in L-Ascorbate Dissimilation in *Escherichia coli*

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**The *ula* regulon, responsible for the utilization of L-ascorbate in *Escherichia coli*, is formed by two divergently transcribed operons, *ulaG* and *ulaABCDEF*. The regulon is negatively regulated by a repressor of the DeoR family which is encoded by the constitutive gene *ulaR* located downstream of *ulaG*. Full repression of the *ula* regulon requires simultaneous interaction of the repressor with both divergent promoters and seems to be dependent on repressor-mediated DNA loop formation, which is helped by the action of integration host factor. Two operator sites have been identified in each promoter. Lack of either of the two sets of operators partially relieved the repression of the other operon; thus, each promoter is dependent on the UlaR operator sites of the other promoter to enhance repression. Electrophoretic mobility shift assays with purified UlaR protein and promoter deletion analyses revealed a conserved sequence, present in each of the four operators, acting as a UlaR binding site. Glucose represses the *ula* regulon via at least two mechanisms, one dependent on cyclic AMP (cAMP)-cAMP receptor protein (CRP) and the other (possibly inducer exclusion) independent of it. Glucose effects mediated by other global regulators cannot be ruled out with the present information. Changes in cAMP-CRP levels affected only the expression of the *ulaABCDEF* operon.**

L-Ascorbate is utilized by *Escherichia coli* K-12 under anaerobic conditions through proteins encoded by the *ula* regulon, previously designated *yjf-sga* (23, 31), located at centisome 95.3 (4). This system is formed by two divergently transcribed operons (6), the *ulaG* operon, proposed to encode the L-ascorbate-6-P lactonase (35), and the *ulaABCDEF* operon, encoding the three components of the L-ascorbate phosphotransferase transport system (UlaABC) (35) and three catabolic enzymes (UlaDEF) (31). The three gene products UlaABC (formerly known as SgaTBA) are involved in the uptake and phosphorylation of L-ascorbate (35). Intracellular L-ascorbate-6-P could be transformed by L-ascorbate-6-P lactonase into 3-keto-L-gulonate-6-P. It has been proposed that this compound is decarboxylated by UlaD to L-xylulose-5-P, which is then converted to D-xylulose-5-P by the sequential actions of UlaE (encoding 3-epimerase activity) and UlaF (encoding 4-epimerase activity) (31). Thus, the function of the gene products of the *ula* system is the transport of L-ascorbate and its transformation into D-xylulose-5-P (31), which is subsequently metabolized by the pentose phosphate pathway.

The *ula* regulon is induced by growth on L-ascorbate and is under the control of the UlaR repressor encoded by the constitutively expressed gene *ulaR* (6). Inactivation of this gene yields the constitutive expression of promoter fusions of the *ula* operons not only under anaerobic conditions but also in the presence of oxygen (6). The *ula* regulon also seems to be controlled by the global regulators cyclic AMP (cAMP)-cAMP receptor protein (CRP) and fumarate nitrate reductase regulator (FNR), since mutations in the corresponding genes abolished or delayed, respectively, growth on L-ascorbate (35).

The UlaR repressor belongs to the DeoR family of bacterial

regulatory proteins (6). Members of this protein family are present in a variety of bacteria, like *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Lactococcus*. Typically, these regulators bind to several operator sites in the promoter regions of the genes controlled. Although these proteins contain highly conserved regions, such as the second helix of the helix-turn-helix motif or a short sequence located 16 or 17 residues upstream of this motif (3), the operator arrangements of some of them are fundamentally different. In the *deo* system of *E. coli*, the repressor binds to multiple widely separated operators with a dyad symmetry sequence (21), whereas in the *glp* system of *E. coli* the repressor binds with high affinity to adjacent, tandemly arranged operators (16, 30, 33). In the case of SpoIIID of *B. subtilis*, the protein binds to several sites that exhibit dyad symmetry, a direct repeat, or an overlapping repeat (34). Furthermore, it has been reported that the cooperative binding of DeoR to several separate operator sites generates a loop in the DNA that is necessary for full repression. The cooperative binding presumably generates a high degree of repression, even though the intrinsic affinity of the repressor for each site is relatively low (1).

Here, we present evidence that full repression of the *ula* regulon requires binding of UlaR to four operator sites and the participation of integration host factor (IHF). We also demonstrate the role of CRP in the direct activation of the *ulaABCDEF* operon and show that *ula* regulon expression was not affected in an FNR mutant.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and phages.** The genotypes and sources of the bacterial strains are given in Table 1. Genetic crosses were performed by P1-mediated transduction (20).

**Growth conditions and preparation of cell extracts.** Cells were grown on Luria broth (LB) or minimal medium and harvested as described previously (5). For anaerobic growth, L-ascorbate or glucose was added to a basal inorganic medium (5) at 20 mM. Casein acid hydrolysate (CAA) was routinely used at 0.5% for

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Description	Source or reference
<b>Strains</b>		
XL1Blue	<i>recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1</i> (F' <i>proAB lacI<sup>q</sup> lacZΔM15 Tn10</i> )	Stratagene
TE2680	F <sup>-</sup> λ <sup>-</sup> IN( <i>rmD-rrmE</i> ) Δ <i>lacX74 rplS galK2 recD::Tn10-tet trpDC700::putA13033::</i> (Kan <sup>r</sup> Cm <sup>r</sup> <i>lac</i> )	10
JMH0317	W3110 <i>rpoS::Tn10</i>	J. Membrillo-Hernandez
JA134	ECL1 Lyx <sup>+</sup>	25
JA182	JA134 Δ( <i>tyrR-fnr-nac-trg</i> )17 <i>zdd-230::Tn9</i>	14
JA184	JA134 <i>himA::cat</i>	14
JA185	JA134 <i>himD3::cat</i>	14
JA188	JA134 <i>rpoS::Tn10</i>	This work
BL21(DE3)	<i>E. coli B F<sup>-</sup> ompT hsdS</i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>gal dcm λ</i> (DE3)	Pharmacia Biotech
JA211	JA134 <i>yiaR::catΔulaR</i>	6
JA216	JA211 <i>crp::cat</i>	This work
<b>Plasmids</b>		
pRS550/pRS551	Ap <sup>r</sup> Km <sup>r</sup> ; contains promoterless <i>lacZYA</i>	28
pET43c (+)	Nus tag <i>bla</i> (Carb <sup>r</sup> ) <i>lacI</i>	Novagen
pET-UlaR	<i>ulaR</i> in pET43c (+)	This work
pBS-CRP	<i>crp</i> in pBluescript	This work

aerobic growth and 1% for anaerobic growth. To increase the cell yield under anaerobic conditions (31), nitrate was added to the cultures at 20 mM. When required, the following antibiotics were used at the concentrations indicated: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 50 μg/ml; carbenicillin, 50 μg/ml; and tetracycline, 12.5 μg/ml. For growth of strains carrying transcriptional fusions, tryptophan was added at 0.1 mM. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropyl-β-D-thiogalactoside) were used at 30 and 10 μg/ml, respectively.

Cell extracts were prepared as described previously (5). The protein concentration was determined by the method of Lowry et al. (18) with bovine serum albumin as a standard.

**Enzyme activities.** β-Galactosidase activity was assayed by hydrolysis of o-nitrophenyl-β-D-galactopyranoside and expressed in Miller units (20). For this purpose, cell cultures were grown until late exponential phase unless otherwise specified. The data reported are a representative set from at least four separate experiments performed in duplicate.

**DNA manipulation and site-directed mutagenesis.** Bacterial genomic DNA was obtained as described by Silhavy et al. (27). Plasmid DNA was routinely prepared by the boiling method (13). For large-scale preparation, a crude DNA sample was purified on a column (Qiagen). DNA manipulations were performed essentially as described by Sambrook and Russell (24). DNA was sequenced using an automated ABI 377 DNA sequencer and fluorescent dye termination methods. DNA fragments were amplified by PCR using *E. coli* chromosomal DNA as a template. When necessary, specific restriction sites were incorporated at the 5' ends of the primers to facilitate cloning of the fragments in the appropriate vector. PCRs were performed with *Pfu* DNA polymerase under standard conditions. All primers used will be provided upon request.

Site-directed mutagenesis of UlaR and cAMP-CRP binding sites in probes used in the mobility shift assays was performed by PCR using primers containing the desired mutations. Site-directed mutagenesis of the cAMP-CRP binding site and insertion of 5 or 10 bp in the fragment containing the full-length *ulaG* and *ulaA* promoter region were done by crossover PCR (17).

**Isolation of RNA and primer extension.** Total RNA was obtained with a Qiagen RNeasy Total RNA kit from cultures of strain JA211 grown aerobically on CAA. For determination of the 5' ends of the *ulaG* and *ulaA* genes, primer ULA-A, complementary to an internal region of *ulaG*, and primer ULA-A7, complementary to an internal region of *ulaA*, were used. The reactions were performed with 50 μg of total RNA at 37°C for 30 min with 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and [thio-α-<sup>32</sup>S]dATP (>1,000 Ci/mmol; Amersham Pharmacia Biotech) and were followed by a 30-min chase with all four nucleotides (at 1 mM each). As a reference, double-strand sequence reactions were performed with the same primers using the dideoxy chain termination procedure of Sanger et al. (26).

**Construction of lacZ fusions and deletions of the *ulaG* and *ulaA* promoters.** To create operon fusions, DNA fragments of the 5' upstream regions of the *ulaG* and *ulaA* genes were cloned into plasmids PRS551 and pRS550 (28), respectively. The different 5' deletion-containing fragments were obtained by PCR. The

pRS plasmids carried a cryptic *lac* operon and genes that confer resistance to both kanamycin and ampicillin. Recombinant plasmids were selected after transformation of strain XL1-Blue as blue colonies on LB plates containing X-Gal, ampicillin, and kanamycin and were sequenced using the M13 primer to ensure that no mutation was introduced. Single-copy fusions on the *E. coli* chromosome were obtained by the method of Elliot (10) using strain TE2680. The transformants were selected for kanamycin resistance and screened for sensitivity to ampicillin and chloramphenicol. P1 *vir* lysates were made to transduce the fusions into the desired genetic background.

**Expression and purification of UlaR.** UlaR was purified using the NusA gene fusion system, which yields a construct with a His<sub>6</sub> tag at the carboxy-terminal end of the NusA protein and the recognition site for thrombin cleavage upstream of the amino-terminal end of the UlaR. For this purpose, the *ulaR* gene was amplified by PCR with primers ULA-R1 and ULA-R2 and cloned into the *Bam*HI and *Eco*RI restriction sites of plasmid pET-43c (+), yielding plasmid pET-UlaR. Primer ULA-R1 was designed to fuse the ATG start codon of *ulaR* in frame with the gene encoding NusA. Overproduction of UlaR was achieved in strain BL21(DE3) carrying the recombinant plasmid pET-UlaR after induction by IPTG (1 mM) in LB-carbenicillin medium for 3 h at 37°C, and the NusA fusion protein was purified by affinity chromatography with Ni<sup>2+</sup>-nitrilotriacetic acid resin (Qiagen). For UlaR purification, the cell pellet from a 10-ml culture of strain BL21(DE3) bearing plasmid pET-UlaR was suspended in 0.5 ml of 50 mM sodium phosphate buffer, pH 8.0, containing 0.3 M NaCl and 5 mM 2-mercaptoethanol (buffer A) and was sonicated on ice. The cell lysate was centrifuged at 15,000 × g, and the supernatant was incubated with 0.5 ml of Ni<sup>2+</sup>-nitrilotriacetic acid resin for 1 h with gentle shaking. After the mixture was loaded onto a column, the resin was first washed with 50 mM sodium phosphate buffer, pH 6.0, containing 0.3 M NaCl, 5 mM 2-mercaptoethanol, and 10% glycerol (buffer B). Stepwise elution was performed with buffer B containing 100 and 250 mM imidazole to eliminate contaminant proteins. The bound protein was digested in the column with thrombin (9 U) after incubation at room temperature for 12 h. The cleaved UlaR protein was eluted with buffer A.

**DNA binding studies.** For electrophoretic mobility shift assays, several PCR-amplified fragments were used as probes. These fragments were purified from acrylamide gels and labeled with T4 polynucleotide kinase and [γ-<sup>32</sup>P]ATP (3,000 Ci/mmol; Amersham Pharmacia Biotech).

Electrophoretic mobility shift assays were performed with crude extracts obtained as described by Nunoshiba et al. (22) or with purified UlaR protein. Acrylamide gels containing 10% glycerol were run at 4°C by using 1× Tris-borate-EDTA buffer (2). Protein samples were mixed with <sup>32</sup>P-end-labeled DNA substrates (final concentration, ca. 2.5 nM; 10,000 to 25,000 cpm) in a 20-μl reaction volume containing 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM MgCl<sub>2</sub>, 10% glycerol, and 2 mM dithiothreitol. Poly(dI-dC) was used as a non-specific competitor at 500-fold molar excess. Validation of the binding specificity was performed with unlabeled DNA. After incubation for 15 min at 30°C, a 1/6 volume of 6× gel loading buffer (2) was added, and the mixtures were loaded directly onto prerun gels.

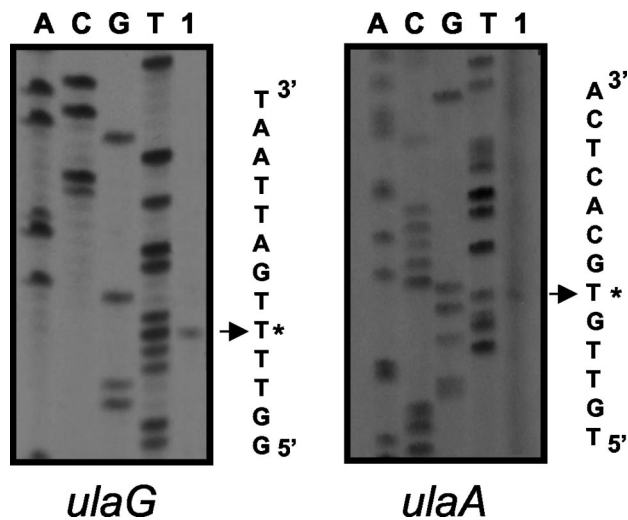


FIG. 1. Primer extension analysis of the divergently transcribed *ulaG* and *ulaA* genes. The primed-extended products using total RNA of strain JA211 growing aerobically on CAA (lanes 1) were electrophoresed with a sequencing ladder (lanes A, C, G, and T) generated by using the same template and primer. A portion of the nucleotide sequence deduced from the sequencing lanes is shown. The extended product assigned as the 5' end of each gene is labeled with an asterisk.

## RESULTS

**Mapping the mRNA 5' ends of *ulaG* and *ulaABCDEF* operons.** The 5' ends of the structural genes *ulaG* and *ulaA* were determined by primer extension analysis. Total RNA was obtained from aerobic cultures on CAA of strain JA211, which constitutively expressed the *ula* regulon due to a mutation in the *ulaR* gene. For the primer extension reactions, we used primers complementary to internal regions of the *ulaG* and *ulaA* genes. The 5' end determined for *ulaG* (Fig. 1) was located 43 bp upstream of the ATG codon. This putative +1 position is preceded by a promoter-like sequence including a -10 box (CAGGAT) and a -35 box (TTGCCT). This promoter region contained an AT-rich sequence between positions -40 and -60. The 5' end determined for *ulaA* (Fig. 1) was located 53 bp upstream of the ATG codon, and the putative +1 was preceded by a promoter-like sequence including a -10 box (TAGAGT) and a -35 box (TTGTTG). This promoter also displayed an AT-rich sequence, in this case between positions -38 and -58. In both cases, the -10 and -35 elements were separated by 17 bp. Using the MacTargsearch program (11), we identified a potential IHF binding site extending between -103 and -150 in the coding strand of *ulaG* and between -102 and -149 in the coding strand of *ulaA*. Sequence analysis also showed four putative CRP binding sites, one centered at -110 with respect to the *ulaG* gene and another centered at -61 with respect to *ulaA*, and two additional sites overlapping the -10 or -35 promoter sequences of the *ulaA* gene (not shown).

**Expression from the functional promoters.** Expression of transcriptional fusions of the two operons encoding the structural genes of the *ula* regulon was analyzed by assaying  $\beta$ -galactosidase activity in strain JA134 carrying  $\Phi(ulaG-lacZ)$  or  $\Phi(ulaA-lacZ)$ . For this purpose, we started an anaerobic culture on L-ascorbate with an aliquot of an overnight culture on

CAA and monitored the growth and  $\beta$ -galactosidase activity. The expression of  $\Phi(ulaA-lacZ)$  was around five times higher than that of  $\Phi(ulaG-lacZ)$  at the end of exponential phase. In both cases, the activity increased through exponential growth, reaching a maximum during transition, and decreased slightly as the culture reached stationary phase (Fig. 2). These results imply that regulation of *ula* regulon expression in *E. coli* occurs at the transcriptional level in a growth phase-dependent manner.

Although during growth most bacterial genes are transcribed by the RNA polymerase complex using the factor  $\sigma^{70}$ , transcription induced under starvation conditions in the stationary phase depends on the factor  $\sigma^s$  encoded by the *rpoS* gene. Since the maximum expression of the *ula* regulon is seen at the transition to stationary phase, we studied the dependence of its expression on  $\sigma^s$ . We transferred, by P1 transduction, the *rpoS* mutation from strain JMH0317 to strain JA134, yielding strain JA188. No differences between the levels of expression of *ulaG* or *ulaA* fusions in the wild type and the *rpoS*-deficient strains were observed in any growth phase (data not shown). The maximum activity was also seen at the end of the exponential phase (Table 2). These results suggest that this sigma factor does not mediate the changes in *ula* regulon expression observed in response to the growth phase.

**Regulation of expression of the *ulaG* and *ulaA* divergent promoters by CRP, IHF, and FNR.** We tested the effect of glucose on the induction of the *ula* regulon by growing strain JA134 carrying  $\Phi(ulaG-lacZ)$  or  $\Phi(ulaA-lacZ)$  on L-ascorbate in the absence or presence of glucose. In the presence of glucose, both transcriptional fusions showed negligible  $\beta$ -galacto-

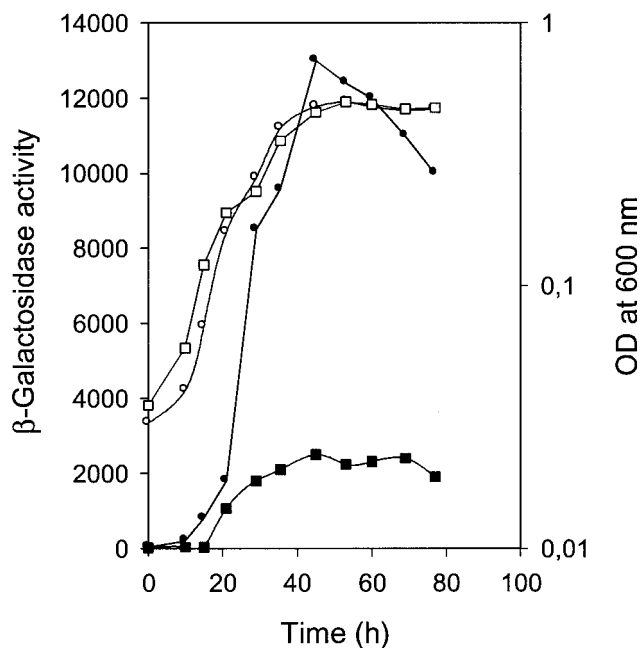


FIG. 2. Expression profiles of  $\Phi(ulaG-lacZ)$  and  $\Phi(ulaA-lacZ)$  transcriptional fusions in strain JA134 growing anaerobically in L-ascorbate. At intervals, the  $A_{600}$  of strain JA134 bearing  $\Phi(ulaG-lacZ)$  (open squares) or  $\Phi(ulaA-lacZ)$  (open circles), as well as the  $\beta$ -galactosidase activities of the corresponding cultures (solid squares and circles, respectively), were measured. OD, optical density.

TABLE 2. Expression analysis of  $\Phi(ulaG-lacZ)$  and  $\Phi(ulaA-lacZ)$  transcriptional fusions in different genetic backgrounds under anaerobic conditions

Strain	Relevant phenotype	$\beta$ -Galactosidase expression <sup>a</sup> (Miller units)			
		$\Phi(ulaG-lacZ)$		$\Phi(ulaA-lacZ)$	
		CAA	L-Ascorbate	CAA	L-Ascorbate
JA134	Wild type	40	2,580	60	12,000
JA184	IHF <sup>-</sup>	130	2,350	2,898	12,260
JA185	IHF <sup>-</sup>	120	2,425	2,940	12,370
JA188	RpoS <sup>-</sup>	55	2,620	60	12,300
JA211	Yjfq <sup>-</sup>	4,850	ND	20,275	ND
JA216	Yjfq <sup>-</sup> CRP <sup>-</sup>	4,720	ND	9,250	ND
JA182	FNR <sup>-</sup>	45	2,650	60	12,375

<sup>a</sup> ND, not determined.

sidase activity, which contrasted with the levels in the absence of glucose (Table 2; also see Fig. 7) and indicated that this sugar produced catabolite repression of the *ula* regulon. The addition of cAMP to a final concentration of 5 mM did not affect glucose repression (see Fig. 7), indicating that the glucose effect is due not only to the reduction of the cAMP-CRP level, as reported by Zhang et al. (35), but also to other mechanisms, such as inducer exclusion, which is mediated by the phosphoenolpyruvate-dependent sugar phosphotransferase.

In order to demonstrate the contribution of cAMP-CRP to the glucose effect on *ula* regulon transcription, we analyzed the expression of  $\Phi(ulaG-lacZ)$  and  $\Phi(ulaA-lacZ)$  in the genetic background of a *crp* mutant, strain JA216. This strain is a JA211 derivative and constitutively expresses *ula* genes, owing to a *ulaR* mutation. Expression of both fusions was analyzed, and only  $\Phi(ulaA-lacZ)$  was reduced around twofold in the *crp* mutant JA216 (Table 2). This indicated that *ulaG* is not affected by CRP and further supports the participation of another mechanism independent of cAMP-CRP in the expression of both operons of the *ula* regulon.

To determine whether IHF influences the divergent promoter expression in vivo, we transferred the operon fusions to the JA134 derivative strains JA184 and JA185, which carried the *himA* and *himD3* mutations, respectively. The  $\beta$ -galactosidase activities showed an increase in the basal level of  $\Phi(ulaG-lacZ)$  and  $\Phi(ulaA-lacZ)$  when cells were grown on CAA (Table 2). These results suggested the participation of IHF in the repression of the *ula* regulon.

Since Zhang et al. (35) had reported that an *fnr* mutant showed delayed growth on L-ascorbate under anaerobic conditions, we tested the effects of an *fnr* mutation on *ula* regulon expression. For this purpose, we transduced  $\phi(ulaG-lacZ)$  and  $\phi(ulaA-lacZ)$  into the *fnr* mutant strain JA182.  $\beta$ -Galactosidase analysis showed that this mutation did not affect the expression of  $\Phi(ulaG-lacZ)$  and  $\Phi(ulaA-lacZ)$  (Table 2), indicating that the delayed growth of this mutant on L-ascorbate is not a consequence of a direct effect of FNR on *ula* regulon expression.

**Binding of UlaR, CRP, and IHF to the *ulaG-ulaA* intergenic region.** The interactions of the regulators UlaR, CRP, and IHF with the promoter regions of the *ula* regulon were studied by electrophoretic mobility shift assays. The repressor interactions with the complete *ulaG-ulaA* intergenic region were assessed using probe P384 (Fig. 3) and UlaR protein, purified as

described in Materials and Methods. As the protein concentration increased, up to three different DNA-UlaR protein complexes were detected (complexes I, II, and III [Fig. 3A]), suggesting that the probe might contain multiple UlaR binding sites, probably with different affinities. When a saturating protein concentration was used, only one complex (complex III) was observed (Fig. 3B), suggesting that this complex is formed when all binding sites are occupied by UlaR. To analyze these plausible multiple DNA binding sites of UlaR, the intergenic region was split into several fragments, which were tested as probes in subsequent experiments with the purified protein. Binding assays performed with probe P194 or P118 (Fig. 4A) displayed in each case up to two complexes, depending on the concentration of protein added to the assay (Fig. 4B), suggesting the presence of two operator sites in each fragment. Sequence analysis of P194 and P118 by OMIGA revealed an extensive similarity in a region of 45 nucleotides, each of which might contain two putative operators (Fig. 4E). The proposed operators were named O<sub>1</sub> and O<sub>2</sub> in the *ulaG* promoter region and O<sub>3</sub> and O<sub>4</sub> in the *ulaA* promoter region. This finding led us to study the binding of UlaR with four probes prepared to contain only one of the operators each (Fig. 4A). As shown in Fig. 4C and D, one complex was formed between UlaR and each DNA probe, indicating that all retained UlaR binding capacity, thus giving support to the function of these operator sites. The addition of L-ascorbate to the binding mixtures did not affect the UlaR binding pattern (not shown), indicating that this compound is not the effector molecule of the UlaR repressor.

Further evidence for the binding of UlaR was obtained by site-directed mutagenesis of the O<sub>2</sub> operator. For this purpose, P115\* was constructed by PCR using primers ULA-A6 and ULA-G4, in which two A's were changed to C (Fig. 4E). Binding experiments showed that UlaR did not bind to this mutated probe (Fig. 4C).

Sequence alignment of the four proposed operators allowed us to define a consensus motif, consisting of an inverted repeat present in each one, which might be recognized by the UlaR repressor (Fig. 5).

Since IHF seems to control *ula* regulon expression and a putative IHF binding site had been identified by computational analysis of the *ulaG-ulaA* intergenic region, we performed gel shift experiments with crude extracts of the wild type and the IHF mutant strain JA184 using probe P100, which contained the putative IHF site (Fig. 6A). The retarded complex observed with wild-type extracts was not present in the IHF mutant, indicating that the transcriptional factor binds to this region (Fig. 6C).

Binding of cAMP-CRP to the the *ulaG-ulaA* intergenic region was undertaken in gel shift experiments performed with different probes and increasing amounts of cell extracts of strain JA211 overexpressing CRP from plasmid pBS-CRP in the presence of various concentrations of cAMP. The results showed that only probe P65, encompassing the putative CRP site located at -61 of *ulaA*, yielded a retarded complex in the presence of cAMP (Fig. 6B). The fact that no additional bands were observed with the other probes when cAMP (100  $\mu$ M to 1 mM) was added to the binding mixtures indicated that no other functional CRP binding sites were present in this region. Moreover, site-directed mutagenesis of the right arm of the

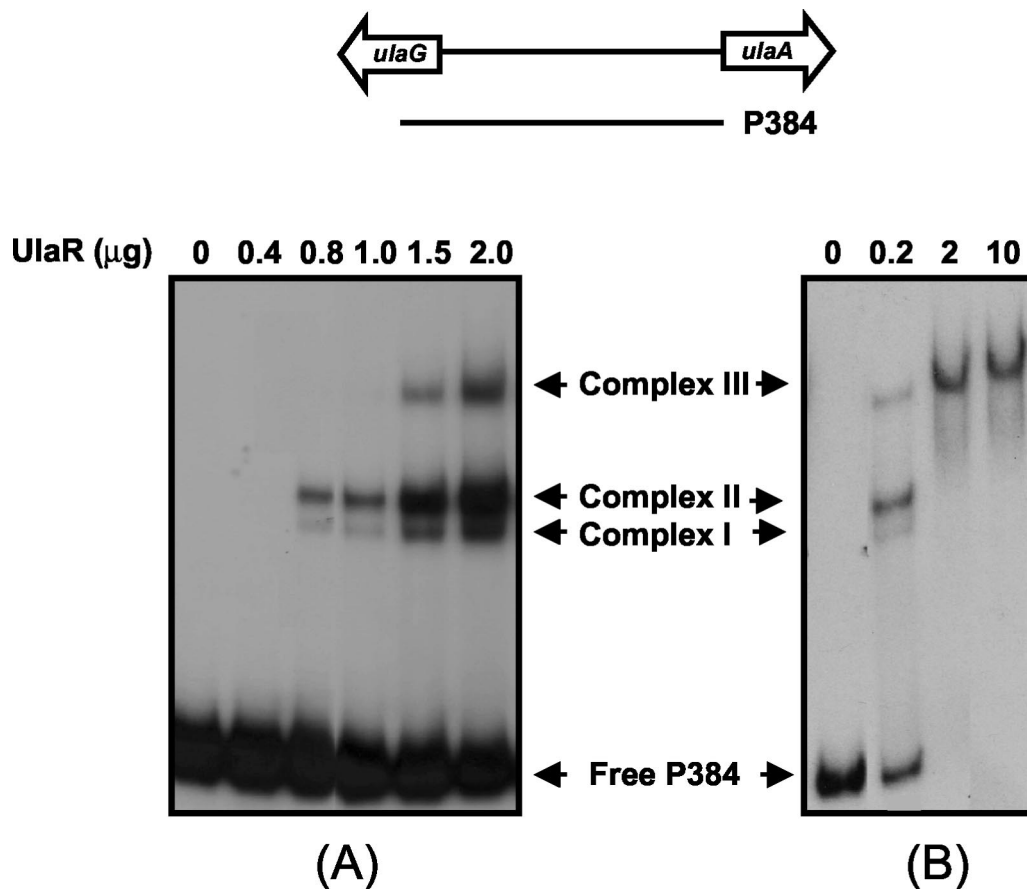


FIG. 3. Binding of UlaR to *ulaG* and *ulaA* promoters. The line over the panels indicates the fragment corresponding to the full-length *ulaG* and *ulaA* promoter region used as a probe. The  $^{32}\text{P}$ -labeled DNA fragment was added at 2.5 (A) or 0.25 (B) nM to binding mixtures containing different amounts of purified UlaR protein. The migration patterns of the different DNA-protein complexes are indicated.

CRP site (Fig. 6A) located at  $-61$  of *ulaA* abolished the cAMP-CRP binding capacity, confirming that this was the only functional CRP site in the region (not shown).

**Deletion analysis of the *ulaG-ulaA* intergenic regulatory region.** To analyze the participation of the identified *cis*-acting motifs in the *in vivo* regulation of the *ulaG* and *ulaABCDEF* operons, we obtained deletion-containing fragments of the corresponding promoters. Those cloned in plasmid pRS551 were used to analyze *ulaG* expression, and those cloned in plasmid pRS550 were used to analyze *ulaA* expression. The *lacZ* fusions obtained were introduced into strain JA134, and  $\beta$ -galactosidase activity was measured after anaerobic growth in CAA in the absence or presence of L-ascorbate.

In the absence of L-ascorbate, a basal level of 60 Miller units was observed for  $\Phi$ -341, corresponding to the full-length promoter of *ulaA* (Fig. 7). Elimination of the  $O_1$  operator site in  $\Phi$ -265 increased the basal level of  $\beta$ -galactosidase activity only  $\sim 3.3$ -fold, whereas the  $O_1$  and  $O_2$  deletion in  $\Phi$ -229 displayed a higher level of uninduced activity (7,500 Miller units). The constitutive expression was maintained in  $\Phi$ -149 and  $\Phi$ -81 and was lost in  $\Phi$ -37. In the last construct, the cAMP-CRP site located at position  $-61$  of the *ulaA* promoter was also deleted. When all the *ulaA* transcriptional fusions were analyzed in CAA in the presence of glucose, 1.5- to 2-fold catabolite repression was seen in the constructions lacking the  $O_1$  or the  $O_1$

and  $O_2$  operator sites. Addition of cAMP partially reversed this repression. These results reinforced the idea that this promoter is regulated by the cAMP-CRP complex.

In the presence of L-ascorbate, *ulaA* expression was induced in  $\Phi$ -341 and  $\Phi$ -265, yielding  $\beta$ -galactosidase levels of  $\sim 12,000$  Miller units. The activity doubled in  $\Phi$ -229 and decreased in  $\Phi$ -81. As under noninducing conditions, deletion to position  $-37$  almost abolished promoter function (Fig. 7). When the *ulaA* transcriptional fusions were analyzed in L-ascorbate plus glucose, 13- to 14-fold repression was seen in the constructions lacking the  $O_1$  and  $O_2$  operator sites, displaying  $\beta$ -galactosidase activities similar to those obtained when glucose was used as the only carbon source (data not shown). Although the addition of cAMP partially reversed the repression, the remaining activity was only  $\sim 15\%$  of the induced value. These results give further support to the previous proposal that glucose repression is also mediated by another mechanism independent of the cAMP-CRP complex.

The analysis of *ulaG* expression showed that under noninducing conditions, the basal level of 40 Miller units displayed by the full-length promoter ( $\Phi$ -288) increased up to 525 Miller units in  $\Phi$ -218, in which the  $O_3$  operator site was totally deleted and  $O_4$  was truncated. Elimination of the cAMP-CRP site in  $\Phi$ -180 did not change the pattern of expression under these conditions. The low level of  $\beta$ -galactosidase activity observed

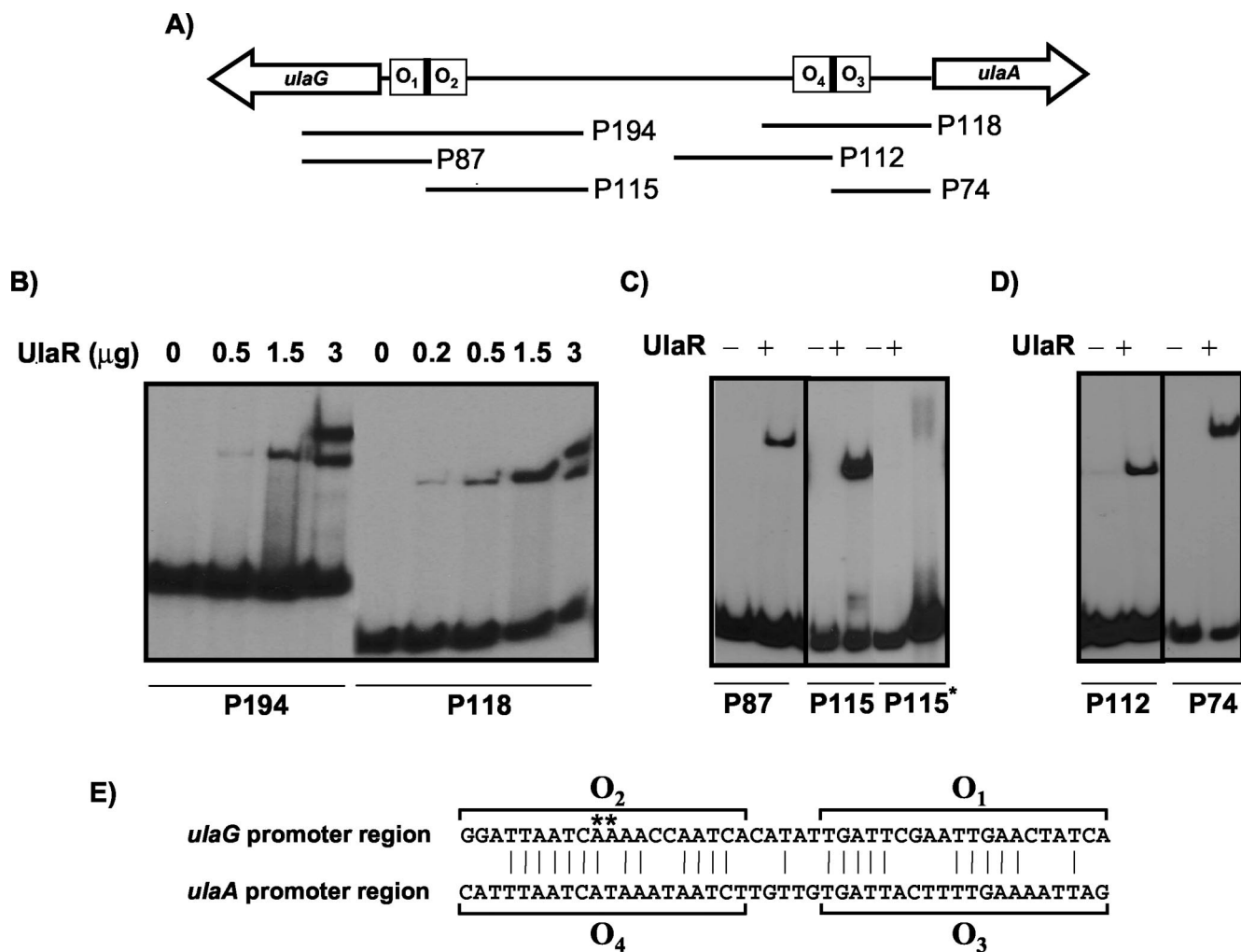


FIG. 4. Binding of UlaR to different fragments of the *ulaG-ulaA* intergenic region. (A) Diagram of *ulaG-ulaA* intergenic region; the fragments used as probes after <sup>32</sup>P labeling are indicated by lines. (B) Binding of UlaR to probes P194 and P118. (C) Binding of UlaR added (+) at 1.5 μg to probes P87, P115, and the mutated P115 (P115\*). (D) Binding of UlaR added at 1.5 μg to probes P112 and P74. (E) Alignment of the nucleotide sequences (written in the direction of transcription of the *ulaG* and *ulaA* genes) of the UlaR binding sites present in fragments P194 and P118. The vertical lines indicate the conserved residues. The nucleotides forming the proposed operator sites are indicated by brackets. Nucleotide changes of the mutation present in P115\* abolishing UlaR binding are shown by asterisks.

with Φ-20 is explained by the deletion of the -35 promoter sequence. No catabolite repression by glucose was seen in any of these constructions.

In L-ascorbate cultures, the induction of *ulaG* was observed as long as the position -67 was retained (Fig. 7). Addition of glucose to these cultures diminished the activity in all constructions to values similar to those obtained under uninduced conditions, and no reversion was observed in the presence of cAMP. These results suggested that the glucose effect on *ulaG* expression is mediated by a mechanism independent of the cAMP-CRP complex.

**Effect of changing DNA helical phasing between the two pairs of UlaR operators.** The results presented above suggest that full repression requires the occupancy of the four operators by UlaR and an additive effect among them that would require the proper orientation. To test whether the repression is affected by the helical phasing of the operators present in the *ulaG* promoter (O<sub>1</sub> and O<sub>2</sub>) and the *ulaA* promoter (O<sub>3</sub> and

O<sub>4</sub>) regions, we changed their angular orientations by inserting 5 bp between the two promoters. The corresponding *ulaG* and *ulaA* transcriptional fusions were analyzed in the corresponding transfected cells grown in CAA, exhibiting basal β-galac-

O <sub>1</sub> operator	TGATTCTGAATTGAACTATCA
O <sub>2</sub> operator	GGATTAATCAAACCAATCA
O <sub>3</sub> operator	TGATTACTTTTGAAAATTAG
O <sub>4</sub> operator	CATTTAATCATAAATAATCT
<b>UlaR Consensus</b>	<b>-GATTA-T--T-AA-AATC-</b>

FIG. 5. Alignment of the nucleotide sequences of the UlaR operator sites. The operator sequences are written in the direction of transcription of the *ulaG* and *ulaA* genes. The nucleotides indicated in the consensus sequence are present in three of the four aligned operators. The arrows indicate the inverted repeat recognized by UlaR.

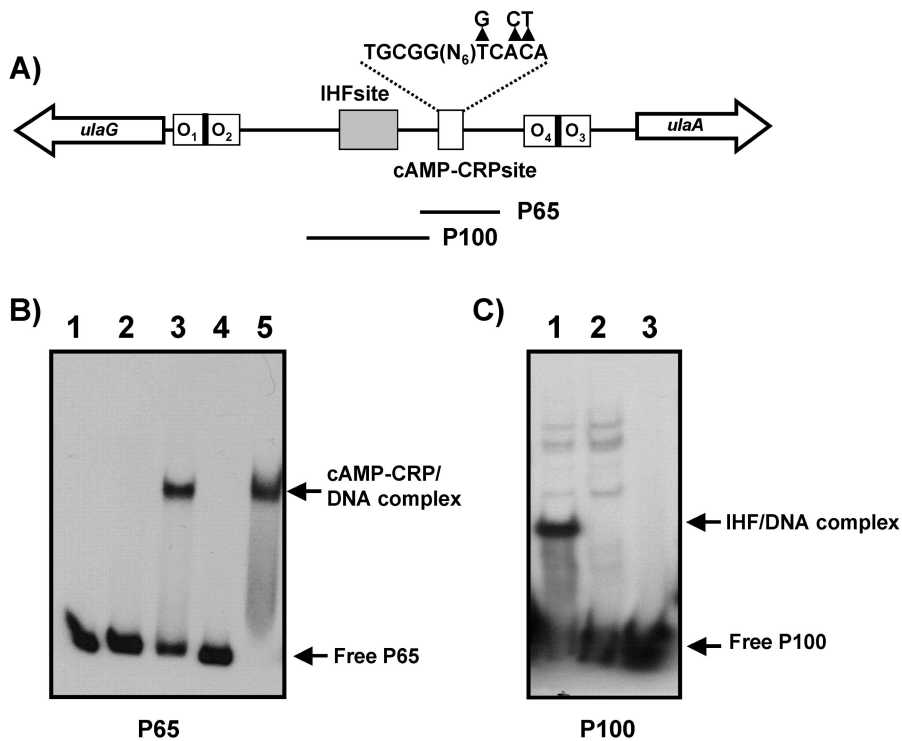


FIG. 6. Interactions of CRP and IHF with different fragments of the *ulaG-ulaA* intergenic region. (A) Location of the putative IHF and CRP binding sites in the intergenic region. The sequence of the CRP site is indicated, and above it the arrowheads indicate the nucleotides changed by site-directed mutagenesis in the CRP site. (B) Binding of cAMP-CRP to probe P65, using 2 (lanes 2 and 3) or 10 (lanes 4 and 5)  $\mu\text{g}$  of crude extracts of strain JA211 transformed with pBS-CRP, in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of 125  $\mu\text{M}$  cAMP. No extract was added to lane 1. (C) Binding of IHF to probe P100 using extracts of strain JA134 (lane 1) and JA184 (lane 2) and no extract (lane 3).

tosidase activities of 45 and 70 Miller units, respectively. Thus, efficient UlaR repression was retained after the helical phasings of both promoters were changed. Similar results were obtained when the inserted sequence was enlarged to 10 bp.

**Expression of *ulaR* under different growth conditions.** In order to study the expression of *ulaR*, the fused promoter  $\Phi(\textit{ulaR-lacZ})$  (6) was introduced in the genetic background of strain JA134. Analysis of  $\beta$ -galactosidase in anaerobic cultures on CAA or L-ascorbate in the presence or absence of glucose yielded no significant differences in the activity level (ca. 300 Miller units). This indicated a constitutive expression of this gene.

## DISCUSSION

The occurrence of multiple repressor binding sites is a rather common phenomenon, as illustrated by the models of the *gal* operon (15, 19) or the *araBAD* operon (9, 12). This is also the case in the DeoR repressor family, which includes, among others, the *deo* operon and the *sn-glycerol-3-P* regulon of *E. coli* (8, 16, 21, 30) and the *iol* operon of *B. subtilis* (32). In these models, a single operon or two divergently transcribed operons are regulated by the cooperative binding of the specific repressor to widely separated operators located in the corresponding promoter regions (7).

In the *ula* regulon, four UlaR operator sites involved in the coordinated regulation of two divergently transcribed operons were identified. A characteristic of the negative control by UlaR is that the protein may bind cooperatively to every one of

the four operators organized in two pairs separated by 187 bp. The pair  $O_1-O_2$  is located in the *ulaG* promoter overlapping the transcription start site and the  $-10$  region, and the pair  $O_3-O_4$  is located in the *ulaA* promoter overlapping the  $-35$  and the  $-10$  promoter sequences. The high constitutive expression of  $\Phi(\textit{ulaA-lacZ})$  only when  $O_1$  and  $O_2$  are totally deleted indicates that repression of the *ulaABCDEF* operon requires interaction of the repressor with these operator sites. The fact that the expression displayed further increase in the presence of L-ascorbate suggests the participation of additional UlaR operator sites in *ulaA* control, a role accomplished by the operators  $O_3$  and  $O_4$  located in the *ulaA* promoter region. In the case of the opposite promoter, deletion of the  $O_3$  and  $O_4$  operator sites also increased the  $\beta$ -galactosidase activity of the  $\Phi(\textit{ulaG-lacZ})$  fusion, although in this case to a lower derepression ratio, as expected from a weaker promoter. The presence of L-ascorbate increased the activity to values probably corresponding to full derepression of the less efficient *ulaG* promoter. Thus, each promoter is dependent on the presence of the operator sites of the other promoter, suggesting that the distant operators are essential to enhance repression. When only one promoter is present, repression can also be obtained with lower efficiency.

Total repression of these operons by UlaR seems to be dependent upon repressor-mediated DNA loop formation. This is especially supported by the fact that a mutation in the *himA* or *himD3* gene encoding the different subunits of the

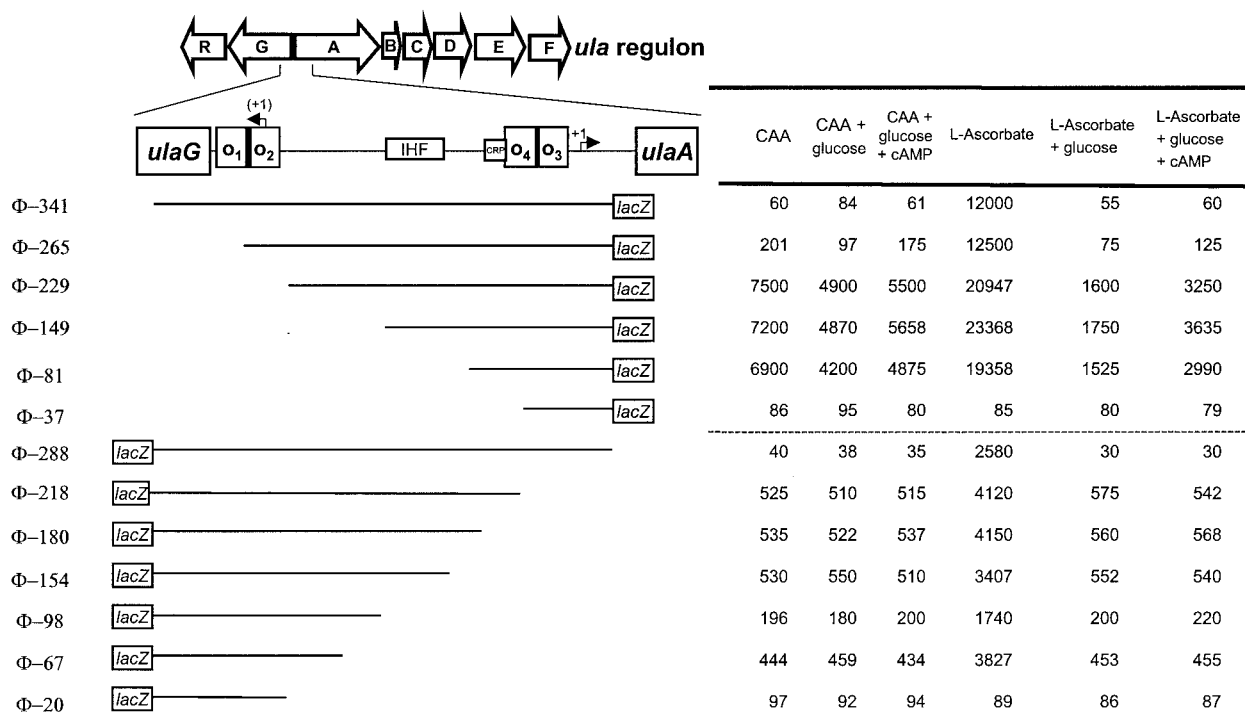


FIG. 7. Effects of upstream deletions on *ulaG* and *ulaA* expression. The open arrows indicate the extents and directions of transcription of the genes included in the *ula* regulon. The regulatory elements recognized by UlaR (*O*<sub>1</sub>, *O*<sub>2</sub>, *O*<sub>3</sub>, and *O*<sub>4</sub>), IHF, and CRP between *ulaG* and *ulaA* are represented by boxes. Deleted promoter fragments are represented by lines, and the corresponding *lacZ* fusions are labeled on the left. The designations of the deletions are based on the number of base pairs present upstream of the 5' end, marked by an arrow labeled +1. The 5' end corresponding to the complementary *ulaG* coding strand is in parentheses. β-Galactosidase activity values under the different growth conditions are indicated in the table on the right.

IHF factor partially relieved the repressor effect in the expression of *ulaG* or *ulaA* promoter fusions when cells grew in the absence of L-ascorbate. This effect was smaller in the case of *ulaG*, probably due to its weaker promoter activity.

As has been proposed for other operons, the DNA looping could increase the local concentration of the UlaR repressor within the promoter regions or alter the structures of the promoter regions in such a way that transcription initiation is no longer possible. In the *ula* regulon, changes in the helical phasing between the two sets of operators do not release the repression, as does the deletion of the pair *O*<sub>3</sub>-*O*<sub>4</sub> in *ulaG* expression or the pair *O*<sub>1</sub>-*O*<sub>2</sub> in *ulaA* expression. A simple explanation is that alternative loops may be formed because of the multiple binding sites. At a given interoperator distance, one specific combination of two of the four binding sites might be most favorable. When the distance is increased by half a helix turn, another combination of two binding sites may be favorable. Formation of different loops had been previously reported to be involved in DeoR regulation (8). This model of UlaR-DNA interaction proposed for the regulation of the divergent *ula* promoters is consistent with the different UlaR-DNA complexes observed in the gel shift experiments. It is noteworthy that, despite its apparent complexity, DNA looping is widely used in gene regulation, and although a well-established role is to increase the repression level, it has recently been reported that compared to a simple alternative regulatory mechanism, DNA looping is able to reduce the fluctuation in

transcription and keep the repression level fairly constant with respect to changes in the number of repressor molecules (29).

The glucose repression of the *ula* regulon is mediated by at least two mechanisms, one independent of cAMP-CRP and the other mediated by this activator complex, which causes a weak, though significant, activation of the *ulaA* operon. Two lines of evidence support the hypothesis of this double mechanism. First, the repression exerted by glucose in Φ(*ulaA-lacZ*) or Φ(*ulaG-lacZ*) expression on the L-ascorbate cultures was not reversed by the addition of cAMP, indicating the occurrence of a mechanism other than that mediated by CRP. Second, in a strain constitutively expressing the *ula* regulon, a *crp* mutation caused a 55% reduction in *ulaA* operon expression, thus requiring another mechanism to account for the full reduction of either *ulaA* or *ulaG*.

In relation to the cAMP-CRP-independent mechanism, since L-ascorbate transport has been reported to be mediated by a phosphotransferase system (35), it seems likely that the phosphorylation of the ascorbate EIIA-specific protein (SgaA) and hence that of L-ascorbate through its specific transport protein. This impairs L-ascorbate transport and therefore the formation of the *ula* regulon inducer. Notice that a most accurate regulation seems to be exerted on the expression of the *ulaA* operon, which contains the genes involved in L-ascorbate transport necessary for the formation of a *ula* regulon inducer. In this context, it must be emphasized that L-ascorbate does



not modify UlaR binding to the operators and that metabolism of L-xylulose-5-phosphate formed endogenously in the cell does not induce the *ula* regulon (6). Therefore, the other two intermediate metabolites in the pathway, L-ascorbate-6-phosphate and 3-keto-L-gulonate-6-phosphate, are the most plausible candidates to act as the effector molecule of the *ula* regulon. Experiments are under way to obtain these compounds, which are not commercially available, to be tested as inducers of the *ula* regulon.

Since addition of glucose to L-ascorbate cultures produced a repression effect on the expression of *ulaA* promoter deletion fusions higher than that observed on CAA, the participation of another global regulator whose expression may depend on the carbon source must be taken into account for *ulaA* regulation.

From our studies and those reported by other authors (16, 21, 30), we may conclude that activation by cAMP-CRP and the repressor effect caused by binding to several operator sites are general features of the genetic systems under the control of repressors belonging to the DeoR family in *E. coli*.

The *ula* regulon undergoes transcriptional regulation as a function of the growth phase, reaching a maximum at the end of exponential phase. We have demonstrated that this activation occurs in an *rpoS*-independent manner. At present, we do not know the biological significance of this expression pattern or the regulators involved in it. More studies need to be performed to further characterize growth phase-dependent *ula* regulon induction.

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