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Identification of the Domains of UreR, an AraC-Like Transcriptional Regulator of the Urease Gene Cluster in *Proteus mirabilis*

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Proteus mirabilis **urease catalyzes the hydrolysis of urea to CO_2 and NH_3, resulting in urinary stone formation in individuals with complicated urinary tract infections. UreR, a member of the AraC family, activates transcription of the genes encoding urease enzyme subunits and accessory proteins, *ureDABCEFG*, as well as its own transcription in the presence of urea. Based on sequence homology with AraC, we hypothesized that UreR contains both a dimerization domain and a DNA-binding domain. A translational fusion of the leucine zipper dimerization domain (amino acids 302 to 350) of C/EBP and the C-terminal half of UreR (amino acids 164 to 293) activated transcription from the *ureD* promoter (p_{ureD}) and bound to a 60-bp fragment containing p_{ureD}, as analyzed by gel shift. These results were consistent with the DNA-binding specificity residing in the C-terminal half of UreR and dimerization being required for activity. To localize the dimerization domain of UreR, a translational fusion of the DNA-binding domain of the LexA repressor (amino acids 1 to 87) and the N-terminal half of UreR (amino acids 1 to 182) was constructed and found to repress transcription from p_{sulA}-*lacZ* (*sulA* is repressed by LexA) and bind to the *sulA* operator site, as analyzed by gel shift. Since LexA binds this site only as a dimer, the $UreR_{1–182}$-$LexA_{1–87}$ fusion also must dimerize to bind p_{sulA}. Indeed, purified UreR-*Myc*-His eluted from a gel filtration column as a dimer. Therefore, we conclude that the dimerization domain of UreR is located within the N-terminal half of UreR. UreR contains three leucines that mimic the leucines that contribute to dimerization of AraC. Mutagenesis of Leu147, Leu148, or L158 alone did not significantly affect UreR function. In contrast, mutagenesis of both Leu147 and Leu148 or all three Leu residues resulted in a 85 or 94% decrease, respectively, in UreR function in the presence of urea ($P < 0.001$). On the contrary, His102 and His175 mutations of UreR resulted in constitutive induction in the absence of urea. We conclude that a dimerization domain resides in the N-terminal half of the polypeptide, that Leu residues may contribute to this function, and that sequences within the C-terminal half of UreR are responsible for DNA binding to the urease promoter regions. Selected His residues also contribute significantly to UreR function.**

Proteus mirabilis infects the urinary tract of humans and is most commonly responsible for causing disease in individuals with structural abnormalities of the urinary tract or in patients who undergo long-term catheterization (16). Cystitis, acute pyelonephritis, and urinary stone formation are all possible consequences of *P. mirabilis* infection (17).

P. mirabilis produces a urea-inducible urease, a high-molecular-weight, multimeric, cytoplasmic nickel metalloenzyme. Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide (18). During the course of infection, the production of ammonia by urea hydrolysis raises the pH in the local environment, subsequently precipitating polyvalent ions that are normally soluble in urine. The result is the formation of urinary stones. The elevated pH also creates an environment that is more favorable for growth of this species (4). Increased ammonia production can also lead to acute inflammation with possible tissue necrosis (18).

The *P. mirabilis* urease gene cluster is found in single copy on the chromosome and consists of eight contiguous genes, *ureRDABCEFG* (12, 19, 24). The *ureA* (UreA, 11 kDa), *ureB* (UreB, 12 kDa), and *ureC* (UreC, 61 kDa) genes encode the structural polypeptides required for the assembly of a catalytically inactive urease apoenzyme (18). The accessory genes, *ureD* (UreD, 31 kDa), *ureE* (UreE, 18 kDa), *ureF* (UreF, 23 kDa), and *ureG* (UreG, 22 kDa), encode proteins required for insertion of nickel ions into the metalloenzyme resulting in catalytically active urease (18). The urease gene cluster is regulated by the gene product of *ureR* (UreR, 33 kDa).

P. mirabilis UreR and the plasmid-encoded UreR found in *Escherichia coli* are positive transcriptional activators of the urease genes. The two proteins share 70% amino acid identity (6) and are functionally interchangeable in the activation of transcription from the *ureR* (p_{ureR}) and *ureD* (p_{ureD}) promoters in both the *P. mirabilis* and plasmid-encoded urease gene clusters (6). The UreR binding sites of both promoters have the consensus sequence T(A/G)(T/C)(A/T)(T/G)(C/T)T(A/T)(T/A)ATTG (25). Both UreR proteins have been shown to activate transcription from p_{ureD} in the presence of urea (11, 6). In addition, UreR regulates its own transcription in the presence of urea from p_{ureR} in the direction opposite the rest of the gene cluster (6). In the absence of urea induction, H-NS represses *ureR* expression (3). Because UreR activates transcription in a urea-inducible manner, it is hypothesized that UreR binds urea; however, this has not been directly demonstrated.

UreR is a member of the AraC family of transcriptional

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regulators and contains a putative helix-turn-helix in addition to an AraC signature sequence (5, 19). The AraC signature sequence, found within all AraC family members, is a second helix-turn-helix that is hypothesized to also bind DNA (7). Moreover, UreR also contains three conserved leucine residues (Leu147, Leu148, and Leu158) in the same relative location with the same spatial distance relative to each other as in AraC (Leu150, Leu151, and Leu161). These leucine residues are critical for AraC dimerization (23), and we therefore also hypothesize that UreR dimerizes via this mechanism. In the presence of arabinose, AraC uses these three critical leucines for dimerization via an antiparallel coiled-coil in a "knobs-into-holes" manner, as elucidated by X-ray crystallographic studies (23). This coiled-coil is also the primary dimerization face in the absence of arabinose, shown by both size exclusion chromatography and sedimentation velocity analytical ultracentrifugation of an AraC mutant with mutations in Leu150, Leu151, Asn154, and Leu161 (15). A secondary dimerization face in the β barrel of AraC is evident; however, it does not appear to represent the primary means of dimer interaction (15).

AraC contains two separate and independent domains, each with a distinct function, namely, dimerization and DNA binding; UreR is predicted to have similar domains with similar functions. Previously, chimeric proteins containing the two domains of AraC to characterize each of the domain's functions were synthesized (2). The predicted AraC DNA-binding domain was fused to C/EBP, a known eukaryotic transcriptional activator that dimerizes via a leucine zipper. The C/EBP-AraC fusion was found to bind to p_{BAD} and activate transcription (2). The hypothesized AraC dimerization domain was fused to the LexA DNA-binding domain. This fusion was predicted to mimic full-length LexA and demonstrated the need for dimerization in order to repress transcription of genes normally turned off by LexA. A chromosomal transcriptional fusion of p_{sulA} to *lacZ* was repressed in the presence of the AraC-LexA fusion protein. This strategy was used to identify both domains of AraC (2).

In this study, we constructed fusion proteins to identify putative domains of UreR and assign dimerization and DNA-binding functions to each of the domains as well as identifying key amino acid residues involved in dimerization and urea induction.

MATERIALS AND METHODS

Chemicals and enzymes. All enzymes were purchased from Life Technologies (Rockville, Md.) or New England Biolabs (Beverly, Mass.). All chemicals were purchased from Sigma-Aldrich (St. Louis, Mo.) unless otherwise noted. The DIG (digoxigenin) gel shift kit was obtained from Amersham Pharmacia Biotech (Piscataway, N.J.).

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Bacteria were grown either in Luria-Bertani (LB) broth at 37°C with aeration in a shaking incubator (200 rpm) or on LB plates containing 1.5% agar at 37°C. Plates and media were supplemented with the antibiotic chloramphenicol (10 μg/ml), tetracycline (7.5 μg/ml), ampicillin (100 μg/ml), or kanamycin (50 μg/ml).

PCR amplification of DNA used to make fusion proteins. PCR primers are listed in Table 2. The amplification protocol for PCRs was as follows: denaturation, 94°C, 3 min; annealing, 50 to 55°C, 45 s; elongation, 72°C, 1 min; for 30 cycles.

Cloning of PCR products. PCR products were ligated into pCR-BluntII-TOPO (Invitrogen, Carlsbad, Calif.). Inserts were excised using the appropriate restriction enzymes (either *Nco*I-*Bam*HI, *Bam*HI-*Xho*I, *Nco*I-*Xho*I, or *Nco*I-*Hin*dIII) and separated by agarose gel electrophoresis. PCR amplification products for the inserts encoding $C/EBP_{302–350}$-Myc-His and $LexA_{1–87}$-Myc-His were digested with restriction enzymes; Table 2 lists inserts and restriction enzymes used. All constructs use an *Nco*I site to ligate into the vector at the gene sequence encoding the start codon. A *Bam*HI site is at the junction of the gene sequences encoding the domains in both $C/EBP_{302–350}$-$UreR_{164–293}$ and $UreR_{1–182}$-$LexA_{1–87}$. All constructs except the gene sequences encoding UreR-Myc-His, $UreR_{164–293}$-Myc-His, $C/EBP_{302–350}$-Myc-His, $LexA_{1–87}$-Myc-His, L147A-L148-Myc-His, and L147A-L148A-L158A-Myc-His, which use a 3′ *Hin*dIII site, contain a *Xho*I site at the 3′ end for cloning into the expression vector. Inserts were purified using a Qiaquick gel extraction kit (Qiagen) and ligated into either pBAD/MHA or pSE380 (Invitrogen). Plasmids were introduced into the corresponding laboratory strain by $CaCl_2$ transformation (21). The $LexA_{1–87}$-Myc-His gene sequence was ligated into pBAD/MHA to take advantage of the Myc-His epitopes. After transformation, the pBAD vector containing the gene sequence encoding $LexA_{1–87}$-Myc-His was purified using a Qiagen miniprep kit. The plasmid was then used in a PCR with primers to amplify the gene sequence for $LexA_{1–87}$-Myc-His. The PCR product was then cut with *Nco*I and *Xho*I and ligated into the *Nco*I and *Xho*I sites in pSE380.

Construction of p_{ureD}-*lacZ* reporter plasmid. A low-copy-number *ureD-lacZ* fusion reporter plasmid compatible with pBAD-Myc-His was constructed. Primers MOB906 and MOB915 were used to PCR amplify an approximately 4.3 kb DNA fragment from plasmid pΔR10 *ureD-lacZ* (11) under the following conditions: 95°C denaturation, 54°C annealing, and 72°C elongation for 30 cycles, using Vent DNA polymerase in the presence of 1 mM $MgSO_4$. The PCR product was gel purified and ligated to PCR-Blunt (Invitrogen), forming pCC026. pCC026 was digested with *Eco*RI, and the 4.3-kb DNA fragment encoding *ureD-lacZ* was ligated to an *Eco*RI-digested derivative of pACYC184 that had previously been cut with *Pvu*II and religated (thus, it does not encode a functional chloramphenicol acetyltransferase). The resulting recombinant plasmid, pCC042, carries a tetracycline resistance marker and encodes a *ureD-lacZ* transcriptional fusion that is activated in the presence of UreR and urea.

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

Strain/plasmid	Description	Reference or source
E. coli strains		
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 deoR recA 1 endA 1 hsdR17* (r_k^- m_k^+) *phoA supE44* λ $^-$*thi-1 gyrA96 relA1*	
Top10	F^- *mcrA*Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL*(Str^r) *endA1 nupG*	
JL1436	F′ *lacI*^{q} *lacZ*ΔM15::Tn*9*/*lexA71*::Tn*5* *recA*$^+$ *sulA211* (λ*sulA*::*lacZ* cI *ind*$^-$)	2
Plasmids		
pSE380		Invitrogen
pBAD/*Myc*-His A		Invitrogen
pCC042	p_{ureD}-*lacZ* reporter construct	This work
pSE380-lexA		2
pSE380-lexA-C/EBP		2
pCP015	$C/EBP_{302–350}$-$UreR_{164–293}$-pBAD	This work
pCP016	Full UreR-pBAD	This work
pCP018	Full UreR-pSE380	This work
pCP019	$UreR_{1–182}$-$LexA_{1–87}$-pSE380	This work
pCP025	$LexA_{1–87}$-pSE380	This work
pCP026	$C/EBP_{302–350}$-pBAD	This work
pCP029	L147A-L148A UreR	This work
pCP030	L147A UreR	This work
pCP031	L148A UreR	This work
pCP051	$UreR_{164–293}$-pBAD	This work
pCP056	$UreR_{His175Ala}$	This work
pCP059	UreR-Myc-His-pBAD	This work
pCP063	$UreR_{His102Ala}$	This work
pCP071	$UreR_{164–293}$-Myc-His-pBAD	This work
pCP072	$C/EBP_{302–350}$-Myc-His-pBAD	This work
pCP073	$LexA_{1–87}$-Myc-His-pSE380	This work
pCP079	L147A-L148A UreR-Myc-His	This work
pCP086	L158A UreR	This work
pCP088	L147A-L148A-L158A UreR	This work
pCP089	L147A-L148A-L158A UreR-Myc-His	This work

TABLE 2. PCR primers used in this study

Primer no.	Primer sequence	Gene	Enzyme cleaved	Construct
906	5′ CGAAATACGGGCAGACATGG3′			p_{ureD}-*lacZ* reporter (*ureR-ureD* intergenic)
915	5′ AGGCAAGTTCAAAATGAACATGG3′			p_{ureD}-*lacZ* reporter (*ureR-ureD* intergenic)
1126	5′ CCATGGAATACAAACACATACTTTCTTCTAAC 3′	*ureR*	5′ *Nco*I	UreR
				UreR-Myc-His
				$UreR_{1–182}$-$LexA_{1–87}$
				L147A-L148A
				L147A
				L148A
				L147A-L148A-Myc-His
				$UreR_{His175Ala}$
				$UreR_{His102Ala}$
				L158A
				L147A-L148A-L158A
				L147A-L148A-L158A-Myc-His
1128	5′ GGATCCATGAAAGCGTTAACGGCCAGGCAACAA3′	*lexA*	5′ *Bam*HI	$UreR_{1–182}$-$LexA_{1–87}$
1129	5′ CTCGAGCTATGGTTCACCGGCAGCCACACGACCTAC 3′	*lexA*	3′ *Xho*I	$UreR_{1–182}$-$LexA_{1–87}$
				$LexA_{1–87}$
1132	5′ CCATGGAGAAAGCCAAACAGCGCAACGTGGAGACG 3′	C/EBP	5′ *Nco*I	$C/EBP_{302–350}$-$UreR_{164–293}$
				$C/EBP_{302–350}$
				$C/EBP_{302–350}$-Myc-His
1133	5′ GGATCCCAAGGAGCTCTCAGGCAGCTGGCGGAA 3′	C/EBP	3′ *Bam*HI	$C/EBP_{302–350}$-$UreR_{164–293}$
				$C/EBP_{302–350}$
1594	5′ GGATCCTTGCGGATCTTGTGTTATTAGATGAGT 3′	*ureR*	3′ *Bam*HI	$UreR_{1–182}$-$LexA_{1–87}$
1595	5′ GGATCCAATTATGATGAGCCAAAAAATCAGGCG 3′	*ureR*	5′ *Bam*HI	$C/EBP_{302–350}$-$UreR_{164–293}$
1596	5′ CTCGAGTTAAAATACTTTTTTTATTGATTCGTC 3′	*ureR*	3′ *Xho*I	$C/EBP_{302–350}$-$UreR_{164–293}$
				UreR
				L147A and L148A
				L147A
				L148A
				L158A
				L147A-L148A-L158A
				$UreR_{His175Ala}$
				$UreR_{His102Ala}$
				$UreR_{164–293}$
1634	5′ AGCCTCTTTTTTATTGCGGCGGCGGTTTATCACGAA 3′	*ureR*		L147A-L148A
				L147A-L148A-Myc-His
				L147A-L148A-L158A
				L147A-L148A-L158A-Myc-His
1635	5′ TTCGTGATAAACCGCCGCCGCAATAAAAAAGAGGCT 3′	*ureR*		L147A-L148A
				L147A-L148A-Myc-His
				L147A-L148A-L158A
				L147A-L148A-L158A-Myc-His
1636	5′ AGCCTCTTTTTTATTGCGCTGGCGGTTTATCAC 3′	*ureR*		L147A
1637	5′ GTGATAAACCGCCAGCGCAATAAAAAAGAGGCT 3′	*ureR*		L147A
1638	5′ CTCTTTTTTATTTTGGCGGCGGTTTATCACGAA 3′	*ureR*		L148A
1639	5′ TTCGTGATAAACCGCCGCCAAAATAAAAAAGAG 3′	*ureR*		L148A
1646	5′ CCATGGCAAAAGCGTTAACGGCCAGGCAACAA 3′	*lexA*	5′ *NcoI*	$LexA_{1–87}$
1647	5′ CTCGAGCAAGGAGCTCTCAGGCAGCTGGCGGAA 3′	C/EBP	3′ *Xho*I	$C/EBP_{302–350}$
1663	5′ AATCAGGCGATCACTGCTCTAATAACACAAGAT 3′	*ureR*		$UreR_{His175Ala}$
1664	5′ ATCTTGTGTTATTAGAGCAGTGATCGCCTGATT 3′	*ureR*		$UreR_{His175Ala}$
1712	5′ CCATGGCAAATTATGATGAGCCAAAAAATCAGGCG 3′	*ureR*	5′ *Nco*I	$UreR_{164–293}$
				$UreR_{164–293}$-Myc-His
1729	5′AAGCTTAAATACTTTTTTTATTGATTCGTC 3′	*ureR*	3′ *Hin*dIII	UreR-Myc-His
				L147A-L148A-Myc-His
				L147A-L148A-L158A-Myc-His
1739	5′ ATTTTACACCGAGTTTCAAAAAATGCGTTTTAAATAAACAGCAAT 3′			p_{ureD} oligo
1740	5′ ATTTTTTCTAAACAAATTGCTGTTTATTTAAAACGCATTTTTTGA3′			p_{ureD} oligo
1749	5′ AATCAATCCAGCCCCTGTGAGTTACTGTATGGATGTACAGTACATCCAGTG 3′			p_{sulA} oligo
1750	5′ TGATCTTTGTTGTCACTGGATGTACTGTACATCCATACAGTAACTCACAGG 3′			p_{sulA} oligo
1797	5′ GCGCCGATTACTCGTGCTCTTCCAGATTATCAT 3′	*ureR*		$UreR_{His102Ala}$
1798	5′ ATGATAATCTGGAAGAGCACGAGTAATCGGCGC 3′	*ureR*		$UreR_{His102Ala}$
1833	5′ TTTTTTAAGCTTAAATACTTTTTTTATTGATTCGTC 3′	*ureR*	3′ *Hin*dIII	$UreR_{164–293}$-Myc-His
1834	5′ TTTTTTAAGCTTTGGTTCACCGGCAGCCACACGACCTAC 3′	*lexA*	3′ *Hin*dIII	$LexA_{1–87}$-Myc-His
1848	5′ AAAAAACCATGGAGAAAGCCAAACA 3′	C/EBP	5′ *Nco*I	$C/EBP_{302–350}$-Myc-His
1851	5′ AAAAAACCATGGCAAAAGCGTTAACGGCCAGGCAACAA 3′	*lexA*	5′ *Nco*I	$LexA_{1–87}$-Myc-His
1852	5′ TTTTTTCTCGAGATGATGATGATGATGATGGTC 3′	*lexA*	3′ *Xho*I	$LexA_{1–87}$-Myc-His
1855	5′ AAAAAAAAGCTTCAAGGAGCTCTCAGGCAGCTGGCGGAA 3′	C/EBP	3′ *Hin*dIII	$C/EBP_{302–350}$-Myc-His
1883	5′ GGGGTCGATATTGCTAATATTTTTCGT 3′	*ureR*		L158A
				L147A-L148A-L158A
				L147A-L148A-L158A-Myc-His
1884	5′ ACGAAAAATATTAGCAATATCGACCCC 3′	*ureR*		L158A
				L147A-L148A-L158A
				L147A-L148A-L158A-Myc-His

Sequencing. Both strands of plasmid constructs were sequenced across each junction and throughout the insert. Sequencing was done by the Biopolymer Laboratory at the University of Maryland, Baltimore.

β-Galactosidase expression assays. Fresh medium was inoculated with a single colony from LB-agar plates containing the appropriate antibiotics and cultured at 37°C overnight. Overnight cultures were used to inoculate fresh medium. Cultures were monitored until they reached an optical density at 600 nm (OD_{600}) of ~0.4 to 0.6, at which time inducer (isopropyl-β-D-thiogalactopyranoside [IPTG], arabinose, or urea) was added, and cultures were incubated for an additional hour. Cultures were placed on ice, and the OD_{600} was measured. Chloroform (100 μl) and 0.1% sodium dodecyl sulfate (SDS; 50 μl) were added, and cultures were vortexed. The suspension of permeabilized cells (10 μl) was added to 990 μl of Z buffer and 200 μl of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml in H_2O). Timed reactions were stopped with 500 μl of 1 M Na_2CO_3. OD_{420} and OD_{550} measurements were recorded, and Miller units were calculated (20). All constructs were assayed in three or more independent experiments.

Western blot analysis. Overnight cultures were used to inoculate 4 ml of fresh LB medium containing the appropriate antibiotics and allowed to grow to mid-exponential phase. Cultures were induced with the appropriate inducer and incubated for the indicated time. Cultures were placed on ice. OD_{600} was determined, and all cultures were adjusted to the same reading. Bacteria were harvested from 1 ml of culture by centrifugation and resuspended in equivalent amounts of Laemmli sample buffer. Samples were boiled for 5 min and placed on ice. The sample volume listed for each experiment was loaded onto a 3.75% stacking and either a 12.5 or 15% SDS-polyacrylamide gel by the method of Laemmli (13). SDS running buffer was used for electrophoresis. Gels were transferred onto Immobilon P membranes in a transfer chamber containing transfer buffer. Transfer occurred overnight at ~12 V at 4°C. Membranes were blocked in 5% dry milk in 0.1% TTBS (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20) for 1 h. Primary antibodies were added to 0.1% TTBS (anti-LexA, anti-Myc and anti-His, diluted 1:5,000), and membranes were exposed to antibodies for 2 h at room temperature. Membranes were washed in 0.1% TTBS three times for 15 min each. Secondary antibodies (anti-mouse immunoglobulin G coupled to alkaline phosphatase detected anti-Myc and anti-His antibodies; anti-rabbit immunoglobulin G coupled to alkaline phosphatase detected anti-LexA antibodies) were placed in 0.1% TTBS (dilution 1:2,000), and membranes were incubated in the secondary antibodies for 1 h. Membranes were washed three times for 15 min each in 0.1% TTBS and developed with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate dissolved in H_2O (according to the Western blot protocol as described in reference 1).

Gel shift analysis. Gel shift experiments used either a 60-bp (DNA-binding domain study) or a 64-bp (dimerization study) double-stranded oligonucleotide that was synthesized from smaller overlapping oligonucleotide fragments that were allowed to anneal and extend using Vent polymerase. One microliter of each reaction was run on a gel for quantification purposes. Both double-stranded oligonucleotides (3.85 pmol of each) were labeled by the DIG gel shift (Amersham Pharmacia) protocol. Each double-stranded oligonucleotide was diluted to 30 fmol for the binding study.

Overnight cultures were used to inoculate fresh LB medium containing appropriate antibiotics. These cultures were grown to mid-exponential phase. The appropriate inducer molecule was added and incubated further. The cultures were placed on ice, and OD_{600} measurements were taken. OD_{600} for all cultures was adjusted to the same reading by using LB medium. Equivalent volumes of each culture were centrifuged, and the pellet was resuspended in 1 ml of TEN buffer (10 mM Tris, 1 mM EDTA, 0.1 M NaCl [pH 8.0]). Bacterial suspensions were disrupted by passage through a French press (18,000 lb/in^2). Lysates were centrifuged (12,000 × *g*, 10 min, 4°C). Supernatants were collected and used as the extract for the binding assay. The binding assay was done as instructed by the DIG gel shift kit manufacturer (Amersham Pharmacia), using binding buffer, 1 μg of poly(dI-dC), 1 μg of poly-L-lysine, specific extract volume, and 2 μl of the labeled DNA. Binding reactions for pBAD, UreR, $C/EBP_{302–350}$-$UreR_{164–293}$, and UreR-Myc-His also included 100 mM urea (final concentration). The binding reactions were run on a preelectrophoresed 6% native polyacrylamide gel in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) at 90 V for 1.5 h. The gel was placed on a Hybond N+ membrane and transferred for 30 min at 400 mA in 1× TAE buffer. The membrane was developed according to the instructions of the DIG gel shift kit manufacturer (Amersham Pharmacia).

Purification of UreR derivatives. *E. coli* Top10 transformed with pCP016 or pCP088 was grown in Luria broth at 37°C. Expression of UreR-Myc-His_6 was induced with 0.2% arabinose when an OD_{600} of ~0.6 was reached. Following 3 h of induction, cells were collected by centrifugation and lysed by two passages through a French pressure cell (18,000 lb/in^2). Single-step purification of UreR-Myc-His_6 was performed by nickel-chelating nitrilotriacetic acid affinity chromatography. A single polypeptide of approximately 33 kDa was eluted from the column with 250 mM imidazole as seen on a Coomassie blue-stained 12% SDS-polyacrylamide gel (data not shown). In a similar experiment, purified protein was electrophoresed, transferred to a nitrocellulose membrane, and reacted with rabbit antiserum specific for the Myc epitope. Western blot anlysis showed that arabinose-induced *E. coli* Top10 transformed with either pCP016 or pCP088 produced a single species of 33 kDa, consistent with the predicted size for UreR-Myc-His_6 (see Fig. 3). The band was absent from the vector control under identical conditions.

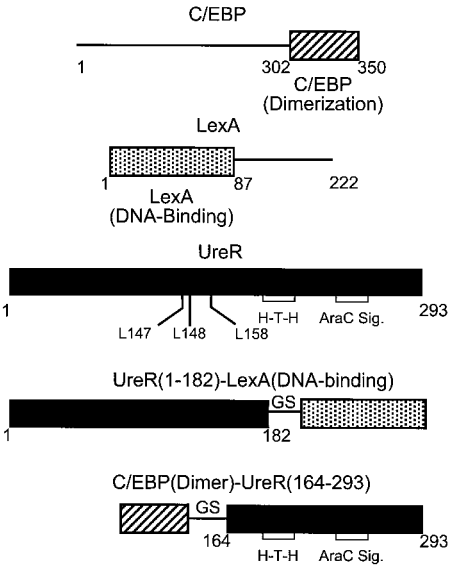

FIG. 1. Schematic of fusion constructs. The domain structure of each of the chimeric proteins is shown; the amino acid residue number is labeled at each boundary. A *Bam*HI restriction site (coding for a Gly-Ser amino acid linker (GS)) was inserted between gene sequences encoding each domain. Key leucine residues and helix-turn-helix motifs (H-T-H) are indicated. Sig., signature.

Gel filtration chromatography. Gel filtration chromatography was performed at room temperature on a Sephadex G-75 column (1 by 35 cm) equilibrated with running buffer (50 mM phosphate [pH 7.5], 150 mM NaCl) at a flow rate of 0.5 ml/min. Molecular weight standards bovine serum albumin (68 kDa) and carbonic anhydrase (34.5 kDa) were used to calibrate the column. Purified wild-type and mutant UreR-Myc-His_6 proteins (100 μl of 0.1 mg/ml) were injected onto the column, and 0.5-ml fractions were collected. The protein elution profile was monitored by absorbance at 280 nm. An aliquot (100 μl) of each fraction was transferred to an Immobilon P membrane, and immunoblot analysis performed with anti-Myc antibodies.

RESULTS

Localization of the UreR DNA-binding domain. To localize the UreR DNA-binding domain, a protein chimera was constructed by fusing the gene sequence encoding the C-terminal half of UreR to the gene sequence encoding the leucine zipper dimerization domain of C/EBP (Fig. 1; primers are listed in Table 2). All translational fusion constructs were cloned into the *E. coli* arabinose-inducible expression vector pBAD/Myc-His A (Invitrogen) (Table 1). A p_{ureD}-*lacZ* plasmid reporter was constructed in pACYC184 to assay specific induction of the urease gene cluster, using β-galactosidase activity as the readout. Both reporter plasmid and pBAD constructs with and without insert were transformed into *E. coli* Top10.

The $C/EBP_{302–350}$-$UreR_{164–293}$ fusion chimera was tested for its ability to bind to p_{ureD} and activate transcription. The $C/EBP_{302–350}$-$UreR_{164–293}$ fusion activated transcription from

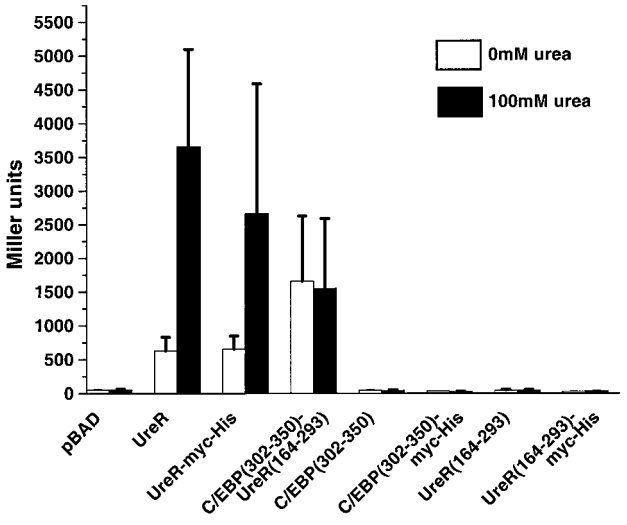

FIG. 2. β-Galactosidase assays using p_{ureD}-*lacZ* reporter construct. β-Galactosidase activity was measured in *E. coli* Top10 transformed with the p_{ureD}-*lacZ* reporter construct and the chimeric fusion constructs. Cultures (4 ml) were grown in LB medium to mid-exponential phase and induced with 0.02% arabinose with or without 100 mM urea for 1 h. β-Galactosidase activity is expressed in Miller units (18). All constructs were assayed in duplicate in at least three different experiments.

this promoter in both the absence (264% of uninduced wild-type UreR level) and presence (42% of urea-induced wild-type UreR level) of urea (Fig. 2). The level of activation of p_{ureD}, mediated by C/EBP$_{302–350}$-UreR$_{164–293}$ and measured by the β-galactosidase assay, was not significantly different in the presence and absence of urea. This result is consistent with the urea-binding domain not residing in the C-terminal half of UreR or urea binding not affecting DNA binding in the chimera.

A UreR-Myc-His fusion was constructed so that UreR could be readily detected on Western blots and in gel mobility shift assays. The UreR-Myc-His fusion induced β-galactosidase expression using the p_{ureD}-*lacZ* reporter construct and elicited levels of expression that were not significantly different from wild-type UreR level (104 and 73% of wild-type UreR activation in the absence and presence of urea, respectively) (Fig. 2). Since we observed similar β-galactosidase expression from p_{ureD}-*lacZ* for both UreR and UreR-Myc-His, we considered these two proteins to be interchangeable in their ability to regulate transcription. This translational fusion contains a c-Myc epitope and His_6 tail that are recognized by anti-Myc (Invitrogen) and anti-His_5 (Qiagen) monoclonal antibodies, respectively. Expression of the UreR-Myc-His protein product was verified by Western blotting. The translational fusion protein was detected as a band corresponding to the expected molecular size of ~36 kDa with both anti-Myc (Fig. 3F) and anti-His (Fig. 3A) antibodies.

Neither the putative UreR DNA-binding domain alone (amino acids 164 to 293) nor C/EBP$_{302–350}$ alone activated transcription from p_{ureD} in the presence or absence of urea; values were below levels detected for the vector plasmid DNA alone (Fig. 2). The stability of UreR$_{164–293}$ and C/EBP$_{302–350}$ was examined by translationally fusing Myc-His to the C-terminal end of both truncated proteins. Both the UreR$_{164–293}$ and the C/EBP$_{302–350}$ proteins showed negligible β-galactosidase activities that were not significantly different from their Myc-His fusion counterparts. Western blot analysis using monoclonal anti-His_5 (Qiagen) revealed that both the UreR$_{164–293}$-Myc-His (18 kDa) and the C/EBP$_{302–350}$-Myc-His (9 kDa) are produced (Fig. 3B and C).

DNA binding by UreR constructs assessed by gel shift. Gel shift experiments were performed to verify that C/EBP$_{302–350}$-UreR$_{164–293}$ used in the β-galactosidase assays could bind to

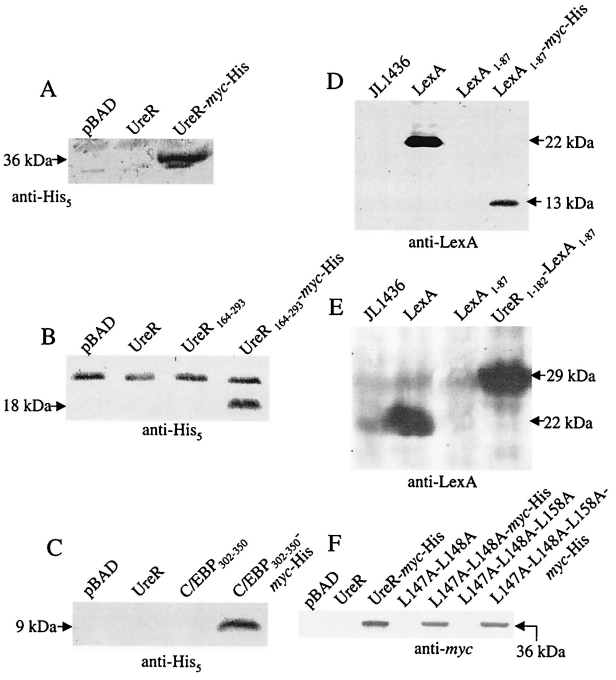

FIG. 3. Western blot analysis of fusion constructs. *E. coli* Top10 (A, B, C, and F) or *E. coli* JL1436 (D and E), transformed with vector or clones expressing UreR or chimeric proteins, was cultured in LB medium to mid-exponential phase and induced with 2% arabinose for 4 h (unless otherwise noted). Samples (10 μl) of each suspension were boiled for 5 min in gel sample buffer and electrophoresed on an SDS-polyacrylamide gel. Membranes were incubated with antiserum or monoclonal antibodies and developed with secondary antibodies conjugated to alkaline phosphotase (Sigma). (A) pBAD, UreR, UreR-Myc-His; 12.5% SDS-polyacrylamide gel. Membranes were reacted with mouse anti-His antibody (diluted 1:1,000) (Invitrogen). (B) pBAD, UreR, UreR$_{164–293}$, or UreR$_{164–293}$-Myc-His; 15% SDS-polyacrylamide gel. Membranes were reacted with mouse anti-His antibody (diluted 1:1,000) (Invitrogen). (C) pBAD, UreR, C/EBP$_{302–350}$, C/EBP$_{302–350}$-Myc-His; 15% SDS-polyacrylamide gel. Membranes were reacted with mouse anti-His antibody (diluted 1:1,000) (Invitrogen). (D) LexA, LexA$_{1–87}$, LexA$_{1–87}$-Myc-His; induced with 2 mM IPTG. Samples (2 μl) of each were electrophoresed on a 15% SDS-polyacrylamide gel. Membranes were hybridized with rabbit polyclonal anti-LexA antibody (diluted 1:5,000) (Invitrogen). (E) LexA, LexA$_{1–87}$, UreR$_{1–182}$-LexA$_{1–87}$; induced with 2 mM IPTG. Samples (1.25 μl) of each, including strain only, were electrophoresed on a 12.5% SDS-polyacrylamide gel. Membranes were reacted with rabbit polyclonal anti-LexA antibody (diluted 1:5,000) (Invitrogen). (F) pBAD, UreR, UreR-Myc-His, UreR L147A-L148A, UreR L147A-L148A-Myc-His, UreR L147A-L148A-L158A, and UreR L147A-L148A-L158A-Myc-His; induced with 2% arabinose for 4 h. Samples (10 μl) of each were electrophoresed on a 12.5% SDS-polyacrylamide gel. Membranes were hybridized with mouse anti-Myc antibody (diluted 1:5,000) (Invitrogen).

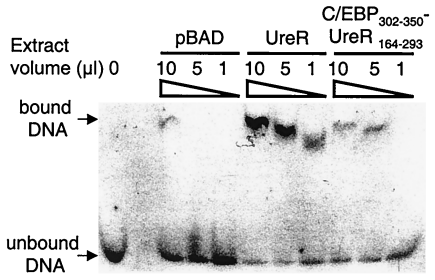

FIG. 4. Gel mobility shift assay for interaction of UreR with the *P. mirabilis* p_{ureD}. A 60-bp double-stranded oligonucleotide was synthesized based on the sequence of p_{ureD}. Whole-cell extracts of *E. coli* Top10 transformed with either pBAD or vector expressing $C/EBP_{302–350}$-$UreR_{164–293}$ were obtained by inducing a 4-ml culture in mid-exponential phase with 2% arabinose for 4 h. Whole-cell extracts of *E. coli* Top10 expressing UreR and UreR-Myc-His were obtained by inducing a 4-ml culture in mid-exponential phase with 2% arabinose and 100 mM urea for 4 h. Bacterial suspensions were adjusted to the same OD, and 3 ml of each culture was harvested by centrifugation (10,000 × *g*, 5 min, 4°C). Bacteria were resuspended in 1 ml of TEN buffer and lysed in a French press (18,000 1b/in^2). Binding reactions with the 60-bp p_{ureD} dsDNA fragment were carried out in the presence of 100 mM urea with various amounts of the extracts.

p_{ureD}. A 60-bp double-stranded oligonucleotide that contained the DNA sequence for p_{ureD} (−66 to −6 upstream of the transcriptional start of *ureD*) was synthesized and labeled with DIG-11-ddUTP by terminal transferase.

Binding assays were performed with the labeled double-stranded oligonucleotide, whole-cell extracts containing each of the overexpressed protein products, and 100 mM urea. A cell extract containing full-length UreR retarded the migration of the double-stranded DNA (dsDNA) fragment, indicating that UreR protein bound to DNA (Fig. 4). The fusion protein, $C/EBP_{302–350}$-$UreR_{164–293}$, also bound the labeled DNA, reflected by a somewhat less intense shifted band. The UreR-Myc-His fusion protein bound and retarded the labeled DNA as well as full-length UreR (data not shown). The pBAD vector control lane showed a faint band at the highest concentration of lysate; however, there are no other bands evident in the pBAD lanes containing lower concentrations of protein. The limited nonspecific binding seen in the vector control lane can likely be explained by the use of whole-cell extracts incubated with the target DNA (14). These results demonstrate that full-length UreR, $C/EBP_{302–350}$-$UreR_{164–293}$, and UreR-Myc-His bind to the p_{ureD} DNA double-stranded oligonucleotide and are likely responsible for the activation from p_{ureD}.

Localization of the UreR dimerization domain. Protein chimeras were constructed to localize the UreR dimerization domain. The gene sequence encoding the N-terminal half (amino acids 1 to 182) of UreR was fused to the gene sequence encoding the LexA DNA-binding domain (amino acids 1 to 87), forming the $UreR_{1–182}$-$LexA_{1–87}$ fusion protein (Fig. 1; primers are listed in Table 2). All fusion constructs used in these experiments were cloned into pSE380 (Invitrogen) (Table 1) under the control of an IPTG-inducible promoter. The reporter strain, JL1436 (Table 1), originally described by Bustos and Schleif (2), consisted of a chromosomal transcriptional fusion of p_{sulA} with *lacZ* placed downstream; the *sulA* gene is repressed by dimerized LexA (2).

The fusion proteins were assayed for the ability to repress transcription of *lac*Z by binding to p_{sulA}. As expected, both untransformed *E. coli* JL1436 and JL1436 transformed with full-length *ureR* showed no repression of β-galactosidase expression (Fig. 5). The $UreR_{1–182}$-$LexA_{1–87}$ fusion repressed transcription from p_{sulA} by factors of 70 and 56 in the absence and presence of urea, respectively, comparable to the results for full-length LexA (the repression factor is defined as the Miller units of the strain only divided by the Miller units of the strain carrying the expression vector). LexA has repression factors of 92 and 127 in the absence and presence of urea, respectively. The LexA DNA-binding domain alone ($LexA_{1–87}$) did not repress, indicated by repression factors of only 8 in the absence of urea and 4 in the presence of urea. As expected, the repression factors seen for both $UreR_{1–182}$-$LexA_{1–87}$ and full-length LexA are not significantly different in the absence of urea, showing that they are similar in the ability to repress transcription from p_{sulA}. However, both LexA and $UreR_{1–182}$-$LexA_{1–87}$ had repression factors that were significantly different ($P \leq 0.001$) from those of the $LexA_{1–87}$ control in the presence and absence of urea, suggesting that the $LexA_{1–87}$ lacks a dimerization domain that was provided by $UreR_{1–182}$ to repress transcription. $UreR_{1–182}$-$LexA_{1–87}$ did not repress transcription in a urea-inducible manner.

We noted that $LexA_{1–87}$ was not stable on Western blots. Thus, to examine whether lack of repression at the *sulA* reporter was due to the lack of stability, we constructed another control. $LexA_{1–87}$ protein was translationally fused to Myc-His at its C-terminal end. $LexA_{1–87}$-Myc-His protein also did not repress transcription from p_{sulA}, as evidenced by β-galactosidase assays. $LexA_{1–87}$, either tagged or not with the Myc-His

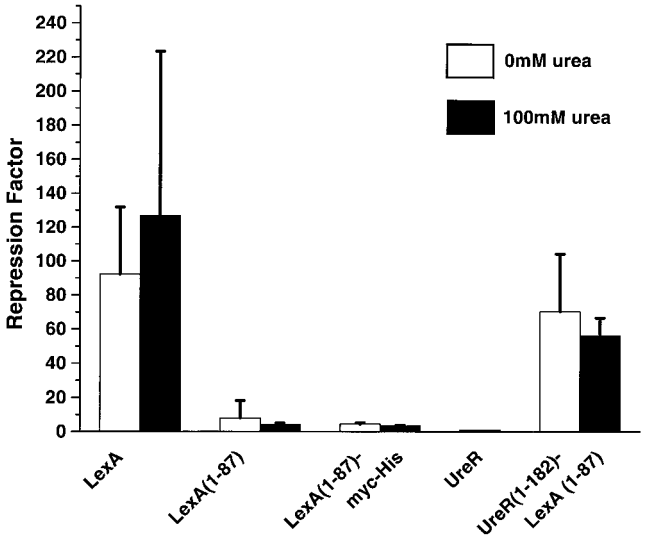

FIG. 5. β-Galactosidase reporter activity of the p_{sulA}-*lacZ* chromosomal fusion reporter construct in strain JL1436. *E. coli* JL1436 (carries a single copy of a p_{sulA}-*lacZ* chromosomal fusion) transformed with the chimeric fusion constructs was cultured in LB medium (4 ml) to mid-exponential phase and induced with 2 mM IPTG with or without 100 mM urea for 1 h. β-Galactosidase levels measured in Miller units (18) as an index of expression from p_{sulA}-*lacZ*. The Repression factor was calculated by dividing the Miller units for strain only (JL1436) by Miller units for each construct in JL1436. All constructs were assayed in duplicate for at least three independent experiments.

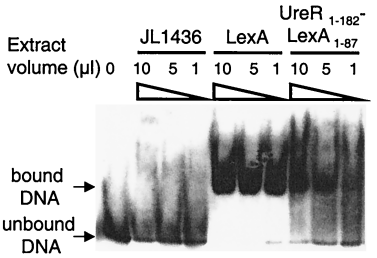

FIG. 6. Gel mobility shift assay for chimeric protein and the LexA DNA-binding site upstream of p_{sulA}. A 64-bp double-stranded oligonucleotide was synthesized based on the nucleotide sequence of p_{sulA}. Whole-cell extracts were obtained by inducing a 100-ml culture, in mid-exponential phase, with 2 mM IPTG for 3 h. Culture (50 ml) was centrifuged, and bacteria were resuspended in 1 ml of TEN buffer and lysed in a French press (18,000 lb/in^2). All lanes contained the labeled 64-bp dsDNA fragment. The lane containing no extract contained labeled DNA only. JL1436, LexA, and $UreR_{1-182}$-$LexA_{1-87}$ whole-cell extracts were added at volumes of 10, 5, and 1 μl.

epitope, showed negligible repression and values were not significantly different.

Expression of both full-length LexA and the $UreR_{1-182}$-$LexA_{1-87}$ used in the β-galactosidase assay was also verified by Western blotting using an anti-LexA polyclonal antibody (Invitrogen) (Fig. 3E). Strong signals were observed in lanes containing LexA and $UreR_{1-182}$-$LexA_{1-87}$. Western blot analysis using anti-LexA showed a 13-kDa band corresponding to $LexA_{1-87}$-Myc-His (Fig. 3D). Therefore, the repression of β-galactosidase expression observed in the $UreR_{1-182}$-$LexA_{1-87}$ β-galactosidase assay was likely due to the expression of the fusion protein.

Binding of the UreR-LexA fusions to target DNA assayed by gel shift. Gel shift experiments were performed to show that the fusion proteins as well as the LexA control bound to p_{sulA}, providing an explanation for the inhibition of transcription initiation from p_{sulA}-*lac*Z. A 64-bp double-stranded oligonucleotide comprising the LexA binding site upstream of *sulA* (+26 to −38 relative to the transcriptional start of *sulA*) was synthesized and labeled with DIG-11-ddUTP by terminal transferase.

Lanes containing LexA revealed a strong signal representing a shifted band (Fig. 6). The $UreR_{1-182}$-$LexA_{1-87}$ lanes contained a retarded band that was not as strong in intensity. Cell extracts from JL1436 carrying no plasmid (Fig. 6) or JL1436 transformed with the $LexA_{1-87}$-Myc-His construct alone (data not shown) were unable to retard the mobility of the dsDNA fragment.

PCR site-directed mutagenesis of leucines in the putative UreR dimerization domain. AraC uses three critical leucines (Leu150, Leu151, and Leu161) for dimerization via an antiparallel coiled-coil in the absence and presence of arabinose (15). Due to the conservation of these three leucines in both overall location and spatial orientation within UreR, we hypothesized that these leucine residues are important for dimerization of UreR. To directly test biologically whether these Leu residues are required for dimerization of UreR monomers, PCR site-directed mutagenesis (9) was used to create leucine to alanine mutants of UreR in Leu147 (L147A) alone, Leu148 (L148A) alone, Leu158 (L158A) alone, Leu147 and Leu148 (L147A-L148A), and Leu147, Leu148, and Leu158 (L147A-L148A-L158A). β-Galactosidase expression from the p_{ureD}-*lac*Z reporter plasmid was measured for each of the UreR leucine mutants in the presence and absence of urea (Fig. 7). In the presence of urea, the L147A, L148A, and L158A single mutants had levels of expression that were 71, 89, and 137%, respectively, of the wild-type UreR level. Thus, dimerization, required for activity, was not dramatically altered. The L147A-L148A double mutant, however, had an expression level that was only 15% of the wild-type UreR level in the presence of urea, a significant drop in activity ($P < 0.001$). The expression level observed for the L147A-L148A-L158A triple mutant, 6% of the wild-type UreR level ($P < 0.001$) in the presence of urea, tended to be even lower than that of the double mutant ($P = 0.052$).

To verify that both the double and triple mutant proteins were expressed and stable, L147A-L148A and L147A-L148A-L158A were translationally fused to Myc-His. The L147A-L148-Myc-His protein activated from p_{ureD} to the same degree (15 and 8% of the wild-type UreR level in the absence and presence of urea, respectively) as seen for L147A-L148A protein in the β-galactosidase assay. The L147A-L148A-L158A-Myc-His protein activated from p_{ureD} to the same degree (11 and 4% of the wild-type UreR level in the absence and presence of urea, respectively) as seen for L147A-L148A-L158A protein in the β-galactosidase assays. Western blot analysis using monoclonal anti-Myc antibodies (Invitrogen) confirmed the stable expression of two 36-kDa bands corresponding to L147A-L148A-Myc-His and L147A-L148A-L158A-Myc-His (Fig. 3F).

Dimer formation in UreR derivatives. To determine whether the low activity of the triple Leu mutant of UreR-Myc-His was due a loss of dimerization, we compared the elution profile

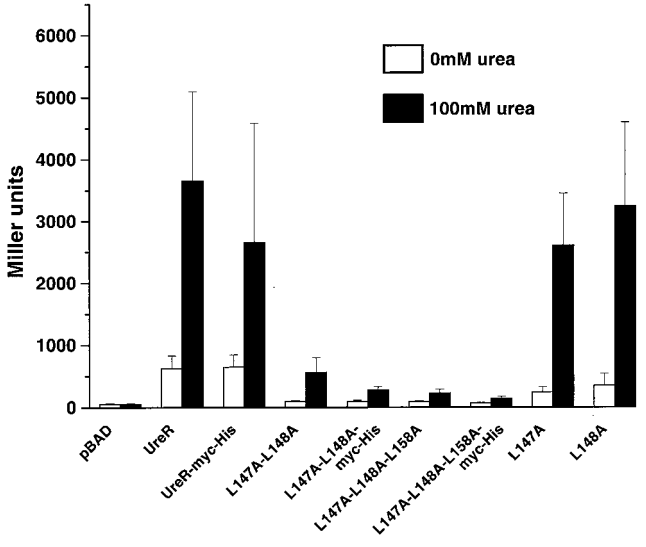

FIG. 7. β-Galactosidase assays using the p_{ureD}-*lacZ* reporter construct to measure activation by the UreR leucine mutants. *E. coli* transformed with the Leu mutant constructs was cultured to mid-log phase in LB medium (4 ml) and induced with 0.02% arabinose with or without 100 mM urea for 1 h. β-Galactosidase activity represented expression from p_{ureD}-*lacZ* and is expressed in Miller units. All constructs were assayed in duplicate for at least three independent experiments.

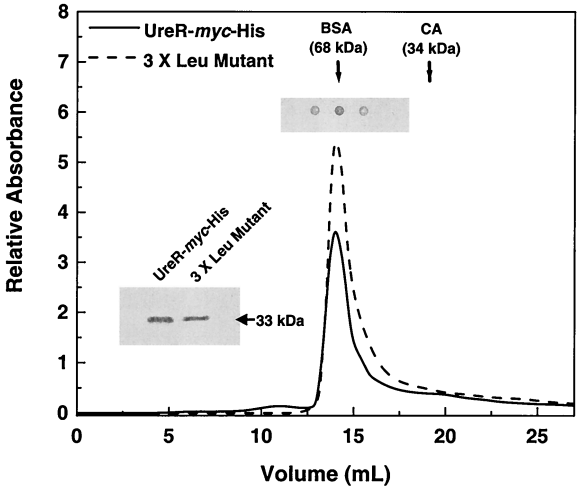

FIG. 8. Gel filtration chromatography of UreR-Myc-His and its triple Leu mutant. Purified UreR-Myc-His and UreR-L147A-L148A-L158A-Myc-His (3 X Leu mutant) (approximately 10 μg of protein) were applied to a Sephadex G-75 column. Protein elution was monitored at 280 nm. Fractions (0.5 ml) were collected, and aliquots of the 3 X Leu mutant (0.15 ml) were assayed by immunoblotting using anti-Myc antibodies (positioned above peak fractions). The elution volumes of bovine serum albumin (BSA) and carbonic anhydrase (CA) are indicated by arrows. The inset shows Western blot of lysates used for purification, run on a denaturing gel, and developed using anti-Myc antibodies. The apparent molecular size is noted.

of this protein to that of UreR-Myc-His on a Sephadex G-75 gel filtration column. The two proteins, purified from induced cell lysates on a Ni-nitrilotriacetic acid column, were applied to the column. Both UreR-Myc-His and the L147A-L148A-L158A-Myc-His derivative of UreR eluted at fractions corresponding to the dimerized protein (Fig. 8). Thus, loss of activity in the site-directed mutant was not due to the inability to dimerize.

Constitutive induction of urease genes by His mutants of UreR. We did not observe urea inducibility using the chimeric fusion proteins. We had reasoned earlier, however, that histidine residues were likely involved in either urea binding or transmission of a structural alteration induced by urea activation to the DNA-binding domain. Indeed, the active site of urease coordinates urea by interaction with four His, one Cys, one Asp, and one Lys residue (18). Thus, urea could be coordinated by similarly configured His residues within UreR. Using site-directed mutagenesis, eight His residues were changed to Ala. Six mutations (H5A, H73A, H107A, H186A, H129A, and H152A) did not significantly alter urea inducibility of the mutated UreR (data not shown). Two of eight His-to-Ala mutants tested, however, H102A and H175A, constitutively induced urease genes (i.e., in the absence of urea), as assayed using the *ureD-lacZ* translational fusion to levels that were not significantly different from the wild-type UreR level in the presence of urea (Table 3). The His102 residue resides in the N-terminal domain, the region predicted to bind urea. Interestingly, the His175 residue resides in the linker region that joins the dimerization domain and DNA-binding domain.

DISCUSSION

UreR chimeric proteins were constructed to identify and localize functional domains of the AraC-like transcriptional activator of the *P. mirabilis* urease gene cluster. Our studies led us to conclude that the N-terminal half of UreR contains the dimerization domain and the C-terminal half of UreR serves as the DNA-binding domain. Leucine residues in the putative dimerization domain of UreR, conserved with respect to AraC and other UreR homologues, were required to fully activate transcription. Site-directed mutagenesis studies were consistent with their involvement in dimerization. While it is proposed that urea binding by UreR is required for activation, construction of the chimeric proteins did not allow us to elucidate this role. Two His mutants of UreR, however, displayed constitutive induction of urease genes.

Our experimental results support the hypothesis that the dimerization domain of UreR localizes to the N-terminal half of UreR. Repression of p_{sulA}-*lacZ* by $UreR_{1-182}$-$LexA_{1-87}$ is consistent with the presence of a dimerization domain supplied by UreR. $LexA_{1-87}$ alone was unable to repress transcription from p_{sulA}-*lacZ* (Fig. 5). Only when the N terminus of UreR was fused to $LexA_{1-87}$ was repression evident, indicating the requirement for dimerization. Other studies have demonstrated that the $LexA_{1-87}$ as well as the λ repressor requires a dimerization domain for full function (8, 10, 22). That dimerization occurs in the fusion protein is further supported by the observation that $UreR_{1-182}$-$LexA_{1-87}$ is capable of retarding the mobility of a double-stranded oligonucleotide containing the *sulA* promoter in a gel shift assay (Fig. 6). $LexA_{1-87}$-Myc-His, a stably expressed protein, does not retard the mobility of the target DNA. Thus, the addition of a dimerization domain, provided by the UreR N-terminal amino acid sequences, to $LexA_{1-87}$ is necessary and sufficient to restore the DNA-binding capability of $LexA_{1-87}$ for its target, p_{sulA}.

A number of observations also led us to conclude that the DNA-binding domain resides in the C-terminal half of UreR. We demonstrated that a functional chimeric protein, $C/EBP_{302-350}$-$UreR_{164-293}$, binds to a double-stranded oligonucleotide containing the mapped UreR-binding site (25) within p_{ureD} (Fig. 4). The UreR-binding site, mapped by Thomas and Collins, is −57 to −34 upstream of the transcriptional start of *ureD* in the *P. mirabilis ureR-ureD* intergenic region (25). Interestingly, $C/EBP_{302-350}$-$UreR_{164-293}$ activated transcription from p_{ureD} to the same degree in both the absence and presence of urea (Fig. 2). This finding is consistent with the hypothesis that the putative urea-binding site resides in the nonhomologous N-terminal domain. The $C/EBP_{302-350}$-$UreR_{164-293}$ fusion has slightly less than optimal activation in comparison to UreR. This may have resulted from constraints imparted on the $UreR_{164-293}$ by the heterologous C/EBP dimerization domain.

TABLE 3. Induction of Urease Genes by His mutants of UreR

E. coli Top10 (p_{ureD}-*lacZ*) cotransformed with:	β-Galactosidase activity (Miller units ± SD)	
	0 mM urea	100 mM urea
pBAD (vector control)	53 ± 15	51 ± 17
pCP016 (UreR)	627 ± 204	3,654 ± 1442
pCP063 ($UreR_{His102Ala}$)	2,414 ± 522[a,b]	4,229 ± 296[c]
pCP056 ($UreR_{His175Ala}$)	2,871 ± 43[a,c]	4,770 ± 262[c]

[a] $P < 0.001$ compared to uninduced (0 mM urea) UreR.
[b] $P = 0.16$, not significantly different from induced (100 mM urea) UreR.
[c] $P > 0.2$, not significantly different from induced (100 mM urea) UreR.

The ability of the C/EBP$_{302–350}$-UreR$_{164–293}$ to bind and activate transcription from p$_{ureD}$ is consistent with DNA-binding specificity residing in the putative UreR DNA-binding domain. This assertion is further supported by the observation that in the absence of a dimerizing mechanism, the UreR DNA-binding domain alone was unable to activate transcription from p$_{ureD}$-*lacZ* even though UreR$_{164–293}$ could recognize its target sequence (Fig. 2) when dimerized. Furthermore, these results suggest that a dimerization domain, provided by C/EBP in this case, is necessary and sufficient to allow the binding of the C-terminal portion of UreR to the *ureD* promoter. Taken together, these results suggest that, as in other AraC family members, dimerization of UreR is required for activation of urease promoters and that the DNA-binding domain, rather than the dimerization domain, resides in the C-terminal portion of UreR.

UreR activates transcription from both p$_{ureD}$ and p$_{ureR}$ in a urea-inducible manner (6), leading to the hypothesis that UreR binds urea. Likely, this mechanism involves a structural change induced by urea binding. We also hypothesized that because the AraC family of transcriptional regulators contains little or no homology in the N-terminal portion of the proteins, binding specificity for an inducer molecule would likely reside in the N-terminal portion of the protein (7). Although UreR$_{1–182}$-LexA$_{1–87}$ represses transcription from p$_{sulA}$-*lacZ*, the hypothesized urea requirement for UreR function was not observed in the β-galactosidase assays (Fig. 5). The tertiary structure of the UreR$_{1–182}$-LexA$_{1–87}$ fusion appears to allow for UreR dimerization to occur but clearly eliminates the urea-inducible mechanism. If UreR dimerizes via two different conformations depending on whether urea is present or not, then possibly only one of the dimerization conformations is attainable when fused to LexA$_{1–87}$, allowing for binding of the UreR$_{1–182}$-LexA$_{1–87}$ fusion to p$_{sulA}$-*lacZ*. This may account for the unresponsiveness of UreR$_{1–182}$-LexA$_{1–87}$ to urea.

Nevertheless, some clues as to the mechanism of urea inducibility by UreR were uncovered by site-directed mutagenesis. Interestingly, among eight His-to-Ala mutants of UreR tested for urea inducibility, two resulted in constitutive transcriptional activation from the *ureD* promoter in the absence of urea. His102 resides in the putative urea-binding (N-terminal) domain. His175 resides in the amino acid sequence that comprises a linker region between the N-terminal and C-terminal domains. It could be speculated that this latter residue transmits the structural alteration that follows urea binding to the C-terminal DNA-binding domain, resulting in binding to specific DNA sequences within the *ureR-ureD* intergenic region.

AraC dimerizes via an antiparallel coiled-coil containing three leucines that fits into a knobs-into-holes conformation at both ends of the coil (23, 15). UreR retains these three conserved leucines in the same spatial orientation and relative location. PCR site-directed mutagenesis of Leu147, Leu148, and Leu158 in UreR demonstrates the requirement of these residues for transcriptional activation of p$_{ureD}$-*lacZ* (Fig. 7). The levels of transcriptional activation for each of the single leucine mutants of UreR are not significantly different from the wild-type UreR level in the presence of urea. The double and triple leucine mutants, however, show a dramatic decrease in activation in comparison to both wild-type UreR and the single leucine mutants in both the presence and absence of urea (as little as 6% of native UreR activity). These results indicate that alteration of each one of the leucines alone is not sufficient to disrupt the protein structure to any degree in the presence of urea. However, because both Leu147 and Leu148 from one monomer may flank Leu158 from the other monomer and act as a dimerization anchor at each end of the coiled-coil helix, the mutation of both Leu147 and Leu148 would be expected to perturb the native dimerization state. A mutation in one of the leucines still may allow for the interaction between the nonmutated leucine and Leu158 from the other monomer. However, mutation of both Leu147 and Leu148 may eliminate the pocket into which Leu158, from the other monomer, is anchored. Surprisingly, while the native dimerization state may have been altered, the triple Leu mutant remained a dimer. The purified Myc-His derivative of the triple mutant eluted at the identical fraction as purified UreR-Myc-His on a gel filtration column (Fig. 8), indicating that under physiologic conditions the two monomers remained associated. More drastic amino acid substitutions of the homologous residues (Leu to Lys in combination with Leu to Ser) in a construct expressing the N-terminal half of AraC did disrupt the dimeric state to yield the monomers (15). Likely, this would be the case for UreR as well. In this study, the Leu residues were conservatively changed to only Ala. While this study is the first to show the biological relevance of these critical leucine residues in UreR, clearly additional studies are required to substantiate their precise role.

The UreR-Myc-His fusion was constructed to detect and follow a protein that mimics wild-type UreR in vivo and in vitro. Although UreR-Myc-His contains the c-Myc and His$_6$ epitopes at the C terminus of UreR, β-galactosidase reporter activity demonstrated that UreR-Myc-His activated transcription from p$_{ureD}$-*lacZ* as well as wild-type UreR (Fig. 2). Overexpression of UreR-Myc-His and detection by anti-Myc and anti-His antibodies on a Western blot show that the fusion protein was produced (Fig. 3). Furthermore, gel mobility shift assays demonstrated that UreR-Myc-His was able to bind to p$_{ureD}$, which resulted in a strong shift of labeled target DNA comparable to the shift seen with wild-type UreR (data not shown).

Using protein fusion technology, we have provided evidence that the AraC-like transcriptional activator UreR consists of dimerization and DNA-binding domains. We have also elucidated a possible dimerization mechanism based on the knobs-into-holes conformation facilitated by three leucine residues that are conserved within both UreR and AraC. Our chimeric proteins were unresponsive to urea activation, and thus a urea-binding domain could not be assigned although we identified two key His residues that were involved in urea inducibility.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1995. Current protocols in molecular biology, p.

10.8.1–10.8.17. John Wiley & Sons, Inc., New York, N.Y.
2. **Bustos, S. A., and R. F. Schleif.** 1993. Functional domains of the AraC protein. Proc. Natl. Acad. Sci. USA **90:**5638–5642.
3. **Coker, C., O. O. Bakare, and H. L. T. Mobley.** 2000. H-NS is a repressor of the *Proteus mirabilis* urease transcriptional activator gene *ureR*. J. Bacteriol. **182:**2649–2653.
4. **Collins, C. M., and S. E. F. D'Orazio.** 1993. Bacterial ureases: structure, regulation of expression and role in pathogenesis. Mol. Microbiol. **9:**907–913.
5. **D'Orazio, S. E. F., and C. M. Collins.** 1993. The plasmid-encoded urease gene cluster of the family *Enterobacteriaceae* is positively regulated by UreR, a member of the AraC family of transcriptional activators. J. Bacteriol. **175:**3459–3467.
6. **D'Orazio, S. E. F., V. Thomas, and C. M. Collins.** 1996. Activation of transcription at divergent urea-dependent promoters by the urease gene regulator UreR. Mol. Microbiol. **21:**643–655.
7. **Gallegos, M., R. Schleif, A. Bairoch, K. Hofmann, and J. L. Ramos.** 1997. AraC/XylS family of transcriptional regulators. Microbiol. Mol. Biol. Rev. **61:**393–410.
8. **Granger-Schnarr, M., E. Benusiglio, M. Schnarr, and P. Sassone-Corsi.** 1992. Transformation and transactivation suppressor activity of the c-Jun leucine zipper fused to a bacterial repressor. Proc. Natl. Acad. Sci. USA **89:**4236–4239.
9. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
10. **Hu, J. C., E. K. O'Shea, P. S. Kim, and R. T. Sauer.** 1990. Sequence requirements for coiled-coils: analysis with lambda repressor-GCN4 leucine zipper fusions. Science **250:**1400–1403.
11. **Island, M. D., and H. L. T. Mobley.** 1995. *Proteus mirabilis* urease: operon fusion and linker insertion analysis of *ure* gene organization, regulation, and function. J. Bacteriol. **177:**5653–5660.
12. **Jones, B. D., and H. L. T. Mobley.** 1989. *Proteus mirabilis* urease: nucleotide sequence determination and comparison with jack bean urease. J. Bacteriol. **171:**6414–6422.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
14. **Lane, D., P. Prentki, and M. Chandler.** 1992. Use of gel retardation to analyze protein-nucleic acid interactions. Microbiol. Rev. **56:**509–528.
15. **LaRonde-LeBlanc, N., and C. Wolberger.** 2000. Characterization of the oligomeric states of wild type and mutant AraC. Biochemistry **39:**11593–11601.
16. **Mobley, H. L. T., and J. W. Warren.** 1987. Urease-positive bacteriuria and obstruction of long-term urinary catheters. J. Clin. Microbiol. **25:**2216–2217.
17. **Mobley, H. L. T., and R. Belas.** 1995. Swarming and pathogenicity of *Proteus mirabilis* in the urinary tract. Trends Microbiol. **3:**280–284.
18. **Mobley, H. L. T., M. D. Island, and R. P. Hausinger.** 1995. Molecular biology of microbial ureases. Microbiol. Rev. **59:**451–480.
19. **Nicholson, E. B., E. A. Concaugh, P. A. Foxall, M. D. Island, and H. L. T. Mobley.** 1993. *Proteus mirabilis* urease: transcriptional regulation by UreR. J. Bacteriol. **175:**465–473.
20. **Platt, T., B. Meuler-Hill, and J. Miller.** 1972. Assays of β-galactosidase activity, p. 352–355. *In* J. Miller (ed.), Experiments in molecular genetics. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
22. **Schmidt-Dörr, T., P. Oertel-Buchheit, C. Pernelle, L. Bracco, M. Schnarr, and M. Granger-Schnarr.** 1991. Construction, purification, and characterization of a hybrid protein comprising DNA binding domain of the LexA repressor and the Jun leucine zipper: a circular dichroism and mutagenesis study. Biochemistry **30:**9657–9664.
23. **Soisson, S. M., B. MacDougall-Shackleton, R. Schleif, and C. Wolberger.** 1997. Structural basis for ligand-regulated oligomerization of AraC. Science **276:**421–425.
24. **Sriwanthana, B., M. D. Island, and H. L. T. Mobley.** 1993. Sequence of the *Proteus mirabilis* urease accessory gene *ureG*. Gene **129:**103–106.
25. **Thomas, V., and C. M. Collins.** 1999. Identification of UreR binding sites in the *Enterobacteriaceae* plasmid-encoded and *Proteus mirabilis* urease gene operons. Mol. Microbiol. **31:**1417–1428.