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Proteus mirabilis Urease: Use of a UreA-LacZ Fusion Demonstrates that Induction Is Highly Specific for Urea

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***Proteus mirabilis*, a common agent of nosocomially acquired and catheter-associated urinary tract infection, is the most frequent cause of infection-induced bladder and kidney stones. Urease-catalyzed urea hydrolysis initiates stone formation in urine and can be inhibited by acetohydroxamic acid and other structural analogs of urea. Since *P. mirabilis* urease is inducible with urea, there has been some concern that urease inhibitors actually induce urease during an active infection, thus compounding the problem of elevated enzyme activity. Quantitating induction by compounds that simultaneously inhibit urease activity has been difficult. Therefore, to study these problems, we constructed a fusion of *ureA* (a urease subunit gene) and *lacZ* (the β-galactosidase gene) within plasmid pMID1010, which encodes an inducible urease of *P. mirabilis* expressed in *E. coli* JM103 (Lac$^-$). The fusion protein, predicted to be 117 kDa, was induced by urea and detected on Western blots (immunoblots) with anti-β-galactosidase antiserum. Peak β-galactosidase activity of 9.9 μmol of ONPG (*o*-nitrophenyl-β-D-galactopyranoside) hydrolyzed per min per mg of protein, quantitated spectrophotometrically, was induced at 200 mM urea. The uninduced rate was 0.2 μmol of ONPG hydrolyzed per min per mg of protein. Induction was specific for urea, as no structural analog of urea (including acetohydroxamic acid, hydroxyurea, thiourea, hippuric acid, flurofamide, or hydroxylamine) induced fusion protein activity. These data suggest that induction by inactivation of UreR, the urease repressor protein that governs regulation of the urease operon, is specific for urea and does not respond to closely related structural analogs.**

Proteus mirabilis, a common cause of nosocomially acquired and catheter-associated urinary tract infection (28), can elicit an acute pyelonephritis and bacteremia (24). The hallmark of *Proteus* urinary tract infections, however, is the development of renal and bladder stones composed of struvite or carbonate-apatite (8). Urease-catalyzed urea hydrolysis elevates ammonia levels in urine, which favors the precipitation of polyvalent cations and anions.

The effects of urease can be combatted clinically by oral administration of specific enzyme inhibitors that act as noncompetitive or competitive inhibitors of urea hydrolysis (7, 23). Many of these compounds, such as acetohydroxamic acid, hydroxyurea, thiourea, and the intermediates of hydrolysis of these compounds (6), act as structural analogs of urea and have similar stereochemical properties, allowing them to bind to the active site of the enzyme (19).

Proteus ureases are induced 5- to 25-fold in the presence of substrate urea (18). Transcription of the enzyme structural genes, *ureA*, *ureB*, and *ureC*, is ordinarily repressed by a *trans*-acting protein encoded by *ureR* (21). The presence of urea derepresses transcription, presumably by binding to UreR, causing the release of the repressor from a putative operator region that precedes the structural genes. Previous studies have suggested that urease inhibitors may compound the problem of high urease activity by preventing the hydrolysis of urea, thus prolonging induction (22). The interpretation of the results of these studies, however, was confounded by the simultaneous enzyme inhibition of the added compound. In other words, low levels of induction would have been difficult to ascertain because of the inhibition of urease itself. We have sought to determine whether urease inhibitors induce urease synthesis by construction of a gene fusion of *ureA* and *lacZ*. β-Galactosidase activity, instead of urease activity, in response to exposure to urea or urease inhibitors, was monitored. Since this construction no longer produced urease, the hydrolysis of inducer (urea) or the binding of the substrate by enzyme was not a factor. We report that induction appears to be exquisitely specific for urea alone and does not occur in the presence of closely related structural analogs.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *P. mirabilis* HI4320 (MR/P and MR/K fimbriated, hemolysin positive, and urease positive) was isolated from a woman with urinary catheter-associated bacteriuria (10). *Escherichia coli* HB101 (F$^-$ *hsdS20 supE44 proA2 leuB6 rpsL20 recA13 lacY1 galK2 thi-1 ara-14 mcrB xyl-5 mtl-1*) was used as a recipient for transformations (1, 15). *E. coli* JM103 (Δ*lac-proAB thi-1 strA endA1 sbcB15 hsdR4 supE* F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]) was used (3) for expression of the UreA-LacZ fusion protein.

Plasmid pMID1010, encoding urease that is inducible with urea, was constructed from a gene bank cosmid clone of *P. mirabilis* HI4320 chromosomal DNA as previously described (11). Plasmid pLKC481, bearing *lacZY* and Kan^r sequences, was obtained from A. Tiedeman and J. Smith (25).

Plasmid DNA isolation. Plasmid DNA was isolated by alkaline sodium dodecyl sulfate (SDS) extraction (2) from cultures (200 ml) of *E. coli* HB101. DNA was purified by centrifugation to equilibrium in cesium chloride-ethidium bromide density gradients (15).

Western blot. Soluble protein (30 μg) from whole-cell French press lysates of *E. coli* JM103 containing plasmids was electrophoresed on an 8% polyacrylamide gel with a

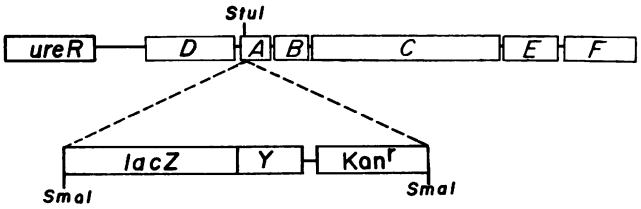

FIG. 1. Construction of the UreA-LacZ fusion protein. The 6.3-kb *lacZY*-Kan^r *Sma*I cartridge from plasmid pLKC481 (25) was cloned into the unique *Stu*I site within *ureA* (which encodes the smallest subunit of urease) of plasmid pMID1010, which encodes an inducible urease of *P. mirabilis* HI4320; the operon spans 5.8 kb (DNA fragments not drawn to scale). The resultant plasmid was designated pMID1501. The fusion protein was predicted to encode the first 17 amino acids of UreA and all but the first 8 of 1,023 amino acids of β-galactosidase.

3.75% stacking gel by the method of Laemmli (13). Polypeptides were transferred to nitrocellulose with a Hoeffer Transphor Power-Lid (model TE50) at 100 V and 500 mA for 4 h. Antisera raised against purified *Morganella morganii* urease (1:50 dilution) (9) or *E. coli* β-galactosidase (1:1,000 dilution) (Sigma) were used for the development of Western blots (immunoblots) by the method of Towbin et al. (27).

Urease activity. Bacteria were inoculated into Luria broth (15) and incubated with aeration at 37°C. When included, urea was added to 200 mM, a concentration shown to give maximal induction of urease (19). At various time points, cells were harvested by centrifugation (10,000 × *g* for 10 min at 4°C), washed once in 20 mM sodium phosphate (pH 6.8), resuspended in 1.7 ml of the sodium phosphate buffer, and ruptured in a French pressure cell (5-ml cell with a 3/8 inch [ca. 0.9 cm] piston) at 20,000 lb/in^2. Lysates (1 ml) were centrifuged in a microcentrifuge for 1 min, and supernatants were assayed directly for urease activity by using the phenol red spectrophotometric assay as previously described (10). The protein concentration was determined by the method of Lowry et al. (14) with bovine serum albumin as a standard. Activities are reported as micromoles of NH_3 liberated per minute per milligram of protein.

Urease inhibitors. Acetohydroxamic acid was obtained from United States Biochemicals. Hydroxyurea, thiourea, hippuric acid, and hydroxylamine were purchased from Sigma. Flurofamide was a gift from Norwich-Eaton Pharmaceuticals.

β-Galactosidase assay. *E. coli* JM103(pMID1501) was grown in Luria broth at 37°C with aeration to an optical density at 550 nm (OD_{550}) of 0.1 (1-cm path length) and induced or not with urea. When the OD_{550} was 0.4, the cell suspension (0.5 ml) was sampled and mixed with 0.5 ml of Z buffer (100 mM sodium phosphate [pH 7.0], 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol) and 2 drops of toluene. The suspension was vortexed for 30 s and then shaken for 45 min at 37°C in an open tube to evaporate the toluene (16). The sample (1 ml) was added to a cuvette (1-cm path length) along with 0.2 ml of ONPG (4 mg/ml), and the change in absorbance at OD_{420} was monitored for approximately 60 min at 23°C. The assay was calibrated with known concentrations of ONPG (0.1 to 5 mM) and hydrolyzed to completion with commercially obtained *E. coli* β-galactosidase (Sigma). Rates were determined from linear portions of the curves, and data are expressed as micromoles of ONPG hydrolyzed per minute per milligram of protein.

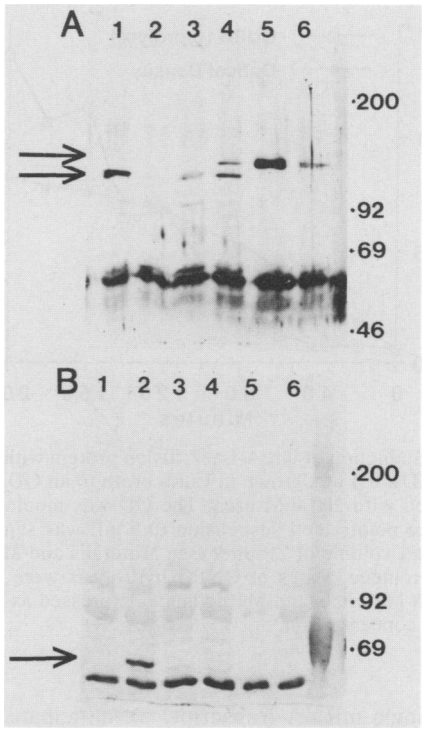

FIG. 2. Western blot of soluble protein from *E. coli* JM103 containing pMID1010 or pMID1501. Soluble protein from *E. coli* JM103 containing pMID1010 (lanes 1 to 3) or pMID1501 (lanes 4 to 6) was electrophoresed on a 7% polyacrylamide gel, transferred to nitrocellulose, and reacted with antiserum directed against β-galactosidase (A) or bacterial urease (B). Samples were derived from cells that were induced with IPTG (lanes 1 and 4) or urea (lanes 2 and 5) or were uninduced (lanes 3 and 6). The arrows in panel A indicate UreA-LacZ fusion protein (upper arrow; apparent molecular weight, 122,000) and inactive native β-galactosidase of strain JM103 bearing a deletion (lower arrow; apparent molecular weight, 112,000). The arrow in panel B indicates UreC polypeptide (molecular weight, 61,000).

RESULTS

Construction of *ureA-lacZ* gene fusions. Plasmid pMID1010, which encodes an inducible urease and was derived from the *P. mirabilis* chromosome by *Bal* 31 deletion of a cosmid gene bank subclone (11), was used for construction of a *ureA-lacZ* gene fusion (Fig. 1). The plasmid was digested with *Stu*I, which cuts uniquely after nucleotide 50 of *ureA* (nucleotide 1336 from reference 12). A *lacZ* cartridge, isolated from pLKC481 (25) by digestion with *Sma*I, was ligated into linearized pMID1010. Transformants were selected on Luria agar containing kanamycin (50 μg/ml). The resultant plasmid of 20.0 kb, designated pMID1501, was isolated, and insertion of the proper fragment was confirmed by agarose gel electrophoresis of various restriction enzyme digests. From nucleotide sequencing data of the urease genes (12), an in-frame fusion protein of UreA and LacZ was predicted.

Evidence of UreA-LacZ fusion protein. The UreA-LacZ fusion protein was predicted to contain the first 17 amino acids of UreA and all but the first 8 of a total of 1,023 amino acids of β-galactosidase (4). The fusion protein, therefore, was predicted to contain 1,032 amino acids and have a molecular size of 117 kDa. Since *ureA*, *ureB*, and *ureC*, the three structural genes of *P. mirabilis* urease, are predicted to

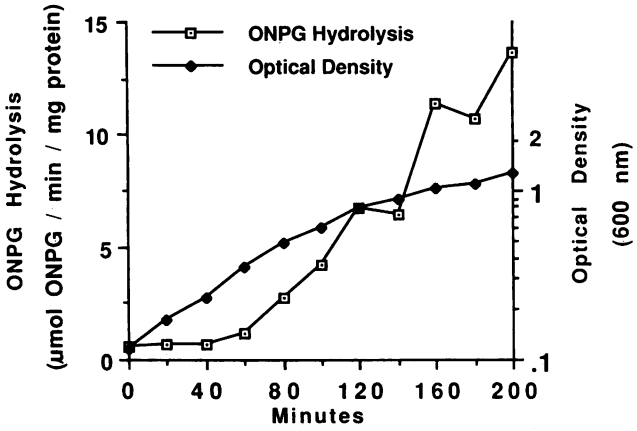

FIG. 3. Induction of UreA-LacZ fusion protein with urea. *E. coli* JM103(pMID1501) was grown in Luria broth to an OD_{550} of 0.1 and then induced with 200 mM urea. The OD was monitored, and, at various time points, cell suspension (0.5 ml) was sampled, mixed with an equal volume of Z buffer (see Materials and Methods), and lysed with toluene. Rates of ONPG hydrolysis were measured as described in Materials and Methods and expressed as a function of the protein concentration.

be on a single mRNA transcript, we anticipated that *ureB* and *ureC* would not be transcribed and thus the corresponding subunit polypeptides would not be synthesized.

Western blots were used to identify the presence or absence of UreC (the largest [61-kDa] subunit polypeptide of urease) and β-galactosidase (Fig. 2). Soluble protein from *E. coli* JM103 containing pMID1010 was electrophoresed on an SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and reacted with mouse anti-β-galactosidase. These lanes revealed the presence of a polypeptide of 112 kDa which was induced by IPTG (Fig. 2A, lane 1). This presumably represents the inactive gene product of the *lacZ* deletion characteristic of strain JM103. We are not aware that this polypeptide has been described. This band was also faintly present in protein samples from uninduced cells (Fig. 2A, lane 3). In cells containing pMID1010, urea induced no additional polypeptides that reacted with anti-β-galactosidase antibodies (Fig. 2A, lane 2).

Protein from cells containing pMID1501 was also reacted with anti-β-galactosidase. Uninduced cells produced a polypeptide corresponding to 122 kDa, a size consistent with the predicted size (117 kDa) of the fusion protein UreA-LacZ. Urea-induced cells produced a much more intense band at this molecular mass, indicating induction of synthesis in the presence of urea (Fig. 2A, lane 5). IPTG (isopropyl-β-D-thiogalactopyranoside)-induced cells produced the basal level of the 122-kDa fusion polypeptide as well as the 112-kDa native deletion LacZ polypeptide (Fig. 2A, lane 4).

To determine whether creation of the *ureA-lacZ* fusion disrupted synthesis of UreC, Western blots of these soluble protein preparations were reacted with antiserum for bacterial urease. Plasmid pMID1010 induced with urea was the only sample to produce UreC, a 61-kDa polypeptide (Fig. 2B, lane 2). As predicted, it was not present in samples from pMID1501 induced with urea (Fig. 2B, lane 5), suggesting that this gene was indeed not transcribed or translated. Presumably, UreB also was not synthesized, but this was not determined.

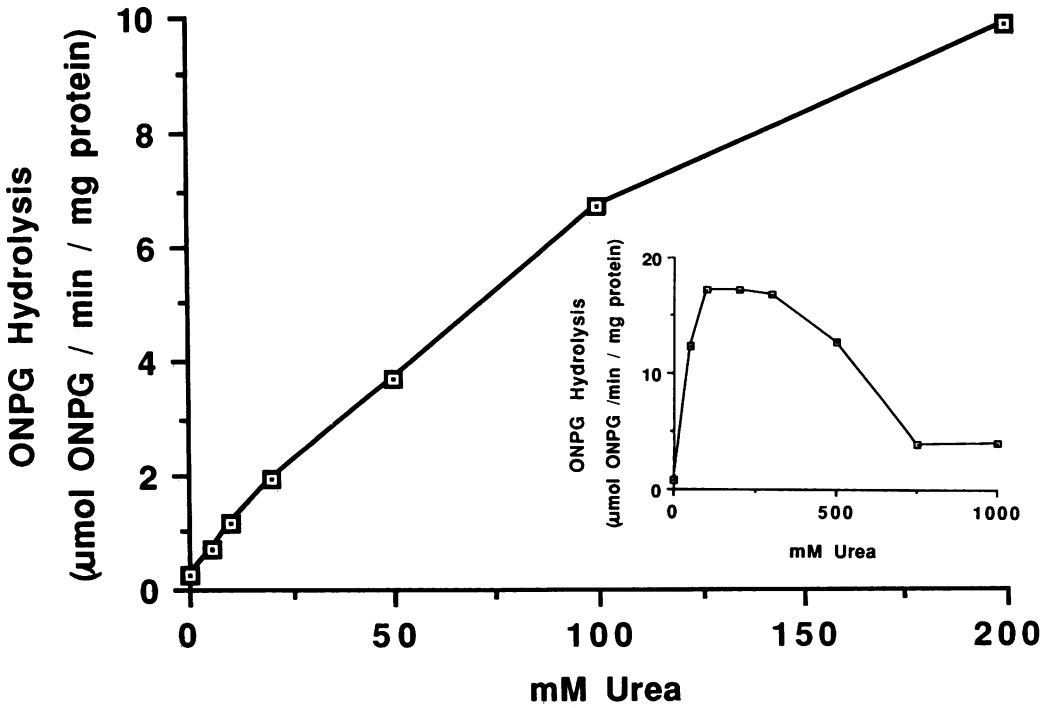

FIG. 4. Induction of UreA-LacZ fusion protein versus urea concentration. *E. coli* JM103(pMID1501) was grown in Luria broth to an OD_{550} of 0.1 and then induced with various concentrations of urea. When cultures reached an OD_{550} of 0.4, cells were harvested and assayed for ONPG hydrolysis as described in Materials and Methods. Rates were expressed as a function of protein concentration. The main and inset graphs depict the results of separate experiments.

Induction by urea. To quantitate induction of the fusion protein by urea, *E. coli* JM103(pMID1501) was grown in Luria broth at 37°C. When the OD_{600} reached 0.1, urea was added to 200 mM and β-galactosidase activity was quantitated by monitoring ONPG hydrolysis (Fig. 3). After a 40-min lag, there was a continuous rise in activity from a baseline of <1 μmol of ONPG hydrolyzed per min per mg of protein up to 13 μmol of ONPG per min per mg of protein in response to urea induction. In the absence of urea, virtually no ONPG hydrolysis (always <1 μmol of ONPG hydrolyzed per min per mg of protein) was observed throughout the experiment (data not shown).

Determination of the optimal urea concentration for induction with wild-type strains or urease clones has been difficult because the inducer (urea) is hydrolyzed and no gratuitous inducer has been identified. However, by using the UreA-LacZ fusion, these studies are straightforward. *E. coli* JM103(pMID1501) was induced with urea when exponentially growing cultures reached an OD_{550} of 0.1. At an OD_{550} of 0.4, cultures were assayed for the rate of ONPG hydrolysis. β-Galactosidase activity rose steadily in response to exposure to urea from 0 to 200 mM, plateaued at 300 and 400 mM urea, and then steadily declined as the concentration of 1 M urea was approached (Fig. 4, inset). Thus, peak induction occurred at urea concentrations normally found in urine (around 400 mM [8]).

Induction experiments were also done with the native urease plasmid pMID1010 (Fig. 5). When no urea was added, barely detectable rates of urease activity could be measured. However, in the presence of 50 or 100 mM urea, peak activities were reached at 40 min; the activities then plateaued and began to decline at between 80 and 100 min. Continuous induction was probably not seen in these experiments because the inducer, urea, was depleted by hydrolysis. This is in contrast to the induction of UreA-LacZ from plasmid pMID1501 (Fig. 3), in which urea was not hydrolyzed and was able to maintain induction for even up to 200 min, at which time bacteria had entered stationary phase.

Lack of induction by urease inhibitors. To test whether common urease inhibitors could induce urease, *E. coli* JM103(pMID1501) was grown in the presence of urea or inhibitor. When urea alone was used as the inducer, the β-galactosidase activity of UreA-LacZ was linearly proportional from 0 to 200 mM (Table 1; Figure 4). Enzyme was induced 42-fold at 200 mM. In contrast, none of the inhibitors shown in Fig. 6, including acetohydroxamic acid, hydroxyurea, thiourea, and hippuric acid, met the definition of induction (a fourfold increase in activity in the presence of inducer) when induced with concentrations ranging from 5 to 200 mM (Table 1, UreA-LacZ columns).

Indeed, in many cases, the β-galactosidase activity of the fusion protein dropped in the presence of higher concentrations of inhibitors. To rule out the inhibition of β-galactosidase itself by inhibitors, purified *E. coli* β-galactosidase (obtained commercially from Sigma) was also assayed in the presence of inhibitor. Inhibitors had no dramatic effect on β-galactosidase activity (Table 1, LacZ columns).

Two other inhibitors were tested at lower concentrations. Flurofamide, a compound that inhibits urease activity at a concentration 1,000-fold less than that of acetohydroxamic acid, was tested at 200, 100, 50, 20, 10, and 5 μM. No induction was observed. Hydroxylamine was tested at 2.5, 1.25, 0.63, 0.32, and 0.16 mM; these concentrations also did not induce the fusion protein. Higher concentrations of hydroxylamine inhibited growth.

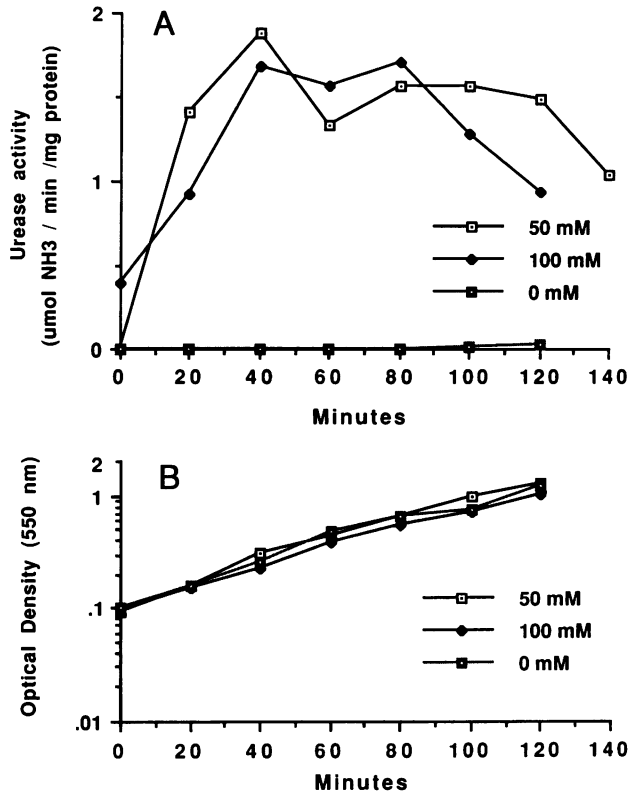

FIG. 5. Induction of urease in *E. coli* HB101(pMID1010) by urea. *E. coli* HB101(pMID1010) was grown in Luria broth at 37°C with aeration to an OD_{550} of 0.1 and then induced with 50 or 100 mM urea. At intervals, cells were harvested, washed, and ruptured in a French press. Soluble protein was assayed for urease activity by the phenol red spectrophotometric assay and expressed as a function of protein concentration.

DISCUSSION

Induction of the *P. mirabilis* urease operon is apparently highly specific for urea. Close structural analogs (acetohydroxamic acid, hydroxyurea, thiourea, and others; Fig. 6) do not allow the derepression of enzyme synthesis even when bacterial cells are grown in very high concentrations of these compounds. The urease of *P. mirabilis* is clearly cytoplasmic, and therefore inhibitory compounds must gain entry into the cell to effectively inhibit the enzyme. Others (17, 19, 22) have shown convincingly that inhibitors (e.g., acetohydroxamic acid, hydroxyurea, and flurofamide) are transported or diffuse into whole cells of *P. mirabilis* and act as potent inhibitors of urease.

We have shown recently (20, 21) that the inducibility of urease synthesis is governed at the transcriptional level by a *trans*-acting protein encoded upstream of six open reading frames (*ureD*, *-A*, *-B*, *-C*, *-E*, and *-F*) that encode the three structural polypeptides and three accessory polypeptides of the urease operon (Fig. 1). This protein encoded by *ureR* has the hallmarks of a repressor protein in that (i) it represses in *trans* in the absence but not presence of urea, (ii) it appears to exist as a dimer, characteristic of many repressors, (iii) it is transcribed in the opposite direction from the rest of the operon, and (iv) it bears similarity at the protein level with a number of *E. coli* regulatory proteins for acid phosphatase synthesis (*appY*), porin synthesis (*envY*), and rhamnose

TABLE 1. Induction of UreA-β-galactosidase fusion protein from *E. coli* JM103(pMID1501) by urea or urease inhibitors

Conc (mM)	Rate of ONPG hydrolysis								
	Urea/UreA-LacZ[a]	Acetohydroxamic acid		Hydroxyurea		Thiourea		Hippuric acid	
		UreA-LacZ	LacZ[b]	UreA-LacZ	LacZ	UreA-LacZ	LacZ	UreA-LacZ	LacZ
0	0.24	0.25	6.19	0.23	5.09	0.24	7.83	0.23	9.88
5	0.73	0.13		0.27		0.16		0.15	
10	1.16	0.08	6.18	0.38	4.81	0.10	7.03	0.18	6.48
20	1.91	0.01		0.17		0.08		0.18	
50	3.66	0.02	6.35	0.17	4.47	0.21	7.64	0.01	7.53
100	6.73	NG[c]	5.59	0.16	4.47	0.05	5.96	0.05	6.08
200	9.85	NG	4.91	0.19	5.01	<.01	5.81	0.09	8.12

[a] Activity of permeabilized, exponentially grown *E. coli* JM103(pMID1501) cultured in the presence of urea or inhibitor. Data are expressed as micromoles of ONPG hydrolyzed per minute per milligram of protein.

[b] Activity of commercially obtained *E. coli* β-galactosidase preincubated with and assayed in the presence of inhibitor. Data are expressed as micromoles of ONPG hydrolyzed per minute per 100 U. As defined by the supplier, 1 U will hydrolyze 1 μmol of ONPG per min at pH 7.3 at 37°C.

[c] No growth of culture.

utilization (*rhaR*). These data allow for a model by which urea binds to the repressor, allosterically changes the conformation of the protein releasing it from a putative operator region, and thus allows transcription of the operon to occur.

The urea binding site on UreR, therefore, must be able to discriminate urea stereochemically from closely related structural analogs. This is in contrast to the catalytic site (located in UreC) of the enzyme itself, which apparently does not make the distinction between urea and analogs,

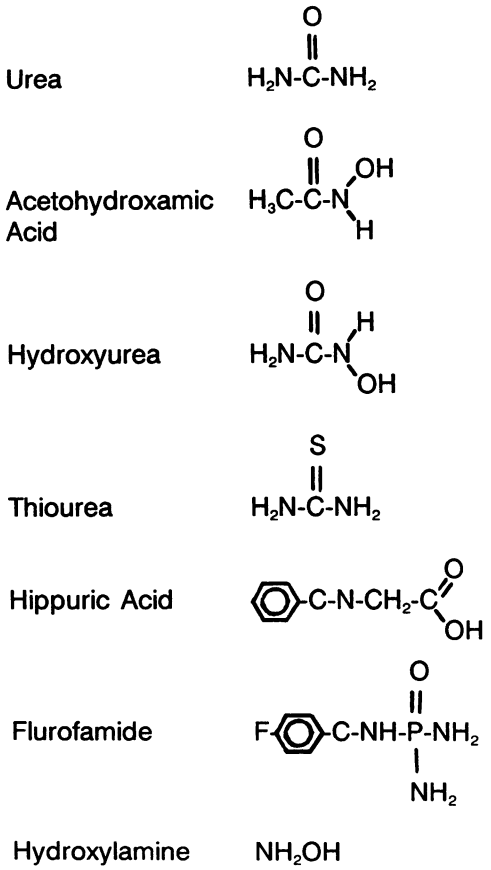

FIG. 6. Chemical structures of urea, structural analogs, and other urease inhibitors used for induction experiments in this study.

since it binds such molecules as acetohydroxamic acid and hydroxyurea (23). Several reports propose mechanisms by which inhibitors coordinate with nickel ions in the active site of bacterial urease (26) and jack bean urease (5). If we assume that UreR binds urea but does not hydrolyze the substrate, it would therefore stand to reason that the predicted amino acid sequences of the repressor UreR and the structural subunit UreC would not necessarily share this sequence homology, even though they both bind urea. Indeed, the amino acid sequences that constitute the putative active site located in UreC (amino acids 308 to 327) are not mimicked by any sequence found in UreR.

The data of Rosenstein et al. (22) which demonstrate that acetohydroxamic acid plus urea give higher levels of induction could be explained by the fact that retardation of urea hydrolysis by the inhibitor maintains a sufficient level of urea to sustain induction of the enzyme for longer periods of time. Since urea is probably not limiting in the urinary tract, acetohydroxamic acid would not function to prolong the induction of urease by *P. mirabilis*; therefore, administration of acetohydroxamic acid should not be discouraged for fear of potentiating urease induction.

An additional conclusion, unrelated to inhibition, can be drawn from these data. Construction of the *ureA-lacZ* fusion supports the premise that *ureA*, *ureB*, and *ureC* are transcribed onto the same mRNA transcript which was predicted by nucleotide sequencing (12). Insertion of the LacZ cassette was found to disrupt synthesis of the large subunit UreC, as assayed by Western blot with antiurease antiserum (Fig. 2B), suggesting that synthesis of this subunit polypeptide is not driven by an independent promoter, an observation consistent with our earlier prediction.

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