JOURNAL OF BACTERIOLOGY, Sept. 1996, p. 5410–5416 Vol. 178, No. 18
0021-9193/96/$04.00+0

Purification, Characterization, and Functional Analysis of a Truncated *Klebsiella aerogenes* UreE Urease Accessory Protein Lacking the Histidine-Rich Carboxyl Terminus

TIMOTHY G. BRAYMAN[1] AND ROBERT P. HAUSINGER[1,2]*

Departments of Biochemistry[1] and Microbiology,[2] Michigan State University, East Lansing, Michigan 48824-1101

Received 10 April 1996/Accepted 17 June 1996

Klebsiella aerogenes **UreE, one of four accessory proteins involved in urease metallocenter assembly, contains a histidine-rich C terminus (10 of the last 15 residues) that is likely to participate in metal ion coordination by this nickel-binding protein. To study the function of the histidine-rich region in urease activation, *ureE* in the urease gene cluster was mutated to result in synthesis of a truncated peptide, H144* UreE, lacking the final 15 residues. Urease activity in cells containing H144* UreE approached the activities for cells possessing the wild-type protein at nickel ion concentrations ranging from 0 to 1 mM in both nutrient-rich and minimal media. In contrast, clear reductions in urease activities were observed when two *ureE* deletion mutant strains were examined, especially at lower nickel ion concentrations. Surprisingly, the H144* UreE, like the wild-type protein, was readily purified with a nickel-nitrilotriacetic acid resin. Denaturing polyacrylamide gel electrophoretic analysis and N-terminal sequencing confirmed that the protein was a truncated UreE. Size exclusion chromatography indicated that the H144* UreE peptide associated into a homodimer, as known for the wild-type protein. The truncated protein was shown to cooperatively bind 1.9 ± 0.2 Ni(II) ions as assessed by equilibrium dialysis measurements, compared with the 6.05 ± 0.25 Ni ions per dimer reported previously for the native protein. These results demonstrate that the histidine-rich motif is not essential to UreE function and is not solely responsible for UreE nickel-binding ability. Rather, we propose that internal nickel binding sites of UreE participate in urease metallocenter assembly.**

UreE is one of four accessory proteins that are thought to participate in the functional assembly of the urease bi-nickel metallocenter (reviewed in reference 15). These proteins are encoded by genes (*ureD*, *ureE*, *ureF*, and *ureG*) that typically are found adjacent to the structural genes (*ureA*, *ureB*, and *ureC*) of urease (reviewed in reference 14). In *Escherichia coli* carrying the *Klebsiella aerogenes* urease gene cluster, deletions within *ureD*, *ureF*, or *ureG* lead to synthesis of inactive urease lacking nickel ions despite the presence of 1 mM $NiCl_2$ in the Luria-Bertani (LB) growth medium (9). In contrast, urease activity is present in *ureE* deletion mutants, and enzyme purified from these cells possesses specific activity values and nickel contents that are 48 to 65% of the levels found for urease purified from cells containing the intact gene cluster (9). These data were interpreted to suggest that UreE is not essential for nickel incorporation into urease but that it somehow facilitates this process. Possibly related to its role in metallocenter assembly, purified *K. aerogenes* UreE reversibly binds approximately six nickel ions per dimer (subunit M_r = 17,558) with an average K_d of ~10 μM (10). Results from X-ray absorption and variable-temperature magnetic circular dichroism spectroscopic examinations of UreE holoprotein indicated the presence of Ni(II) in pseudooctahedral geometry, including coordination by three to five imidazole ligands. Because *K. aerogenes* UreE possesses a carboxyl terminus in which 10 of the last 15 residues are histidines, it is reasonable to speculate that this region of the protein is involved in nickel ion binding. Surprisingly, not all UreE peptides possess a His-rich region (reviewed in reference 15). These findings raise questions concerning the importance of the carboxyl-terminal region in *K. aerogenes* UreE and call into question the postulated functional role for this protein as a nickel donor for urease activation (10).

To assess the importance of the *K. aerogenes* UreE His-rich carboxyl-terminal region and to better understand the function of this protein, we have constructed a plasmid that encodes a truncated UreE protein, H144*, which lacks the last 15 residues. We demonstrate that the absence of the His-rich region has only a small effect on UreE function in urease activation and has no significant influence on the growth rate of cells exposed to various levels of nickel ions. Furthermore, we show that H144* UreE can be purified by nickel-chelate chromatography and that it binds approximately two nickel ions per dimer. Finally, we characterize the ability of the internal nickel ion binding sites to interact with other metal ions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5α (Gibco BRL, Grand Island, N.Y.) was used for routine recombinant DNA work and for most of the urease activity studies. *E. coli* C *slyD* (20), deficient in the production of the His-rich SlyD protein, was used in comparative urease activity studies. For routine cultures, cells were grown overnight at 30 or 37°C in LB liquid or agar medium containing 80 μg of ampicillin (Boehringer Mannheim, Indianapolis, Ind.) per ml and 1 mM $NiCl_2$. When examining the nickel dependence of urease activity or growth rate, the nickel concentrations and times of harvest were varied, and MOPS (morpholinepropanesulfonic acid)-glutamine medium (17) was also used for comparison. Site-directed mutagenesis studies made use of *E. coli* BMH 71-18 *mutS* cells (Clontech, Palo Alto, Calif.) containing pTBEF (see below) grown in LB medium with 80 μg of ampicillin per ml. *E. coli* HMS174 (DE3) cells carrying either of two derivatives of pET21 (cells and vector from Novagen, Madison, Wis.) were used for overproduction of wild-type and H144* UreE proteins. These cultures were grown in LB medium containing 80 μg of ampicillin per ml to an optical density at 600 nm of ~1, induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma Chemical Co., St. Louis, Mo.), and harvested after 3 to 4 h.

Plasmid construction. Plasmid DNA was purified with the Wizard MiniPrep or MidiPrep DNA purification system (Promega, Madison, Wis.). All restriction

* Corresponding author. Mailing address: 160 Giltner Hall, Department of Microbiology, Michigan State University, East Lansing, MI 48824-1101. Phone: (517) 353-9675. Fax: (517) 353-8957. Electronic mail address: RHausing@MSU.EDU.

digestions and common DNA manipulations were performed by standard procedures (21). Plasmid pKAU17 (17) contains the *K. aerogenes ureDABCEFG* genes (9, 16). pKAU17Δ*ureE-1* and pKAU17Δ*ureE-2* are derivatives with deletions in the *ureE* gene (9). pKAUEFG, containing only *ureEFG* of the urease genes, was constructed by digesting pKAU17 with *Eco*RI and *Bsm*I to remove *ureDABC*, treating the 5.5-kbp fragment with the Klenow fragment of *E. coli* DNA polymerase to produce blunt ends, and reannealing with T4 DNA ligase (19). pTBEF was constructed by ligating the 1.8-kbp *Bam*HI-*Avr*II fragment of pKAU17, containing *ureE* and *ureF*, to *Bam*HI- and *Xba*I-digested pUC19 (Gibco BRL). pETH144*, containing a modified *ureE* as well as wild-type *ureF* and *ureG*, was constructed by ligating the *Bam*HI-*Hin*dIII fragment from mutated pKAU17 into pET21 digested with the same enzymes. Additionally, pETWT was constructed by ligating the *Bam*HI-*Hin*dIII fragment from pKAU17, containing *ureEFG*, into pET21 digested with *Bam*HI and *Hin*dIII.

Site-directed mutagenesis. A *ureE* mutation was constructed in pTBEF to contain a stop codon at the position corresponding to His-144, resulting in a translated UreE peptide that would be truncated by 15 residues. In addition to the *ureE* mutagenic oligonucleotide (CCAGCGAGAGC**TAA**GGTCATCATCA TG), a second oligonucleotide (CAGGCATGC**ACGCGT**GGCGTAATC) was used to mutate a *Hin*dIII site to facilitate selection.

Primers were synthesized at the Michigan State University Macromolecular Structure and Sequencing Facility and phosphorylated with T4 kinase (Gibco BRL). Site-directed mutagenesis was accomplished with the Transformer kit (Clontech). Plasmids from recombinant colonies were isolated and screened by *Bam*HI-*Hin*dIII restriction digestion analysis. A 1.2-kbp *Bam*HI-*Aat*II fragment was isolated from those plasmids lacking the *Hin*dIII site, sequenced at the Michigan State University-U.S. Department of Energy Plant Biology Laboratory sequencing facility by dye termination chemistry and an Applied Biosystems automated DNA sequencer, and shown to contain the H144* mutation. The fragment was cloned into the corresponding region of pKAU17 to yield pKAU17H144*. For this construction, we used a pKAU17 whose *Aat*II site in the pUC8 portion of the plasmid had been eliminated by digesting the plasmid with *Aat*II, treating the plasmid with Klenow fragment to produce blunt ends, and reannealing with T4 DNA ligase (19).

Assays. Urease activity was measured by quantifying the rate of ammonia release from urea by formation of indophenol, which was measured at 625 nm (24). Assay buffer contained 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.75), 50 mM urea, and 0.5 mM EDTA. One unit of activity was defined as the amount of enzyme required to hydrolyze 1 μmol of urea per min at 37°C. Protein concentrations were determined by the method of Lowry et al. (12) or with a commercial protein assay (Bio-Rad, Hercules, Calif.) with bovine serum albumin as the standard. In the latter method, an experimentally determined correction factor of 0.79 was used to adjust the values to coincide with the Lowry assay results.

Urease purification. Selected strains were grown overnight in LB medium supplemented with 1 mM $NiCl_2$, harvested by centrifugation, and resuspended in buffer (pH 7.4) containing 20 mM potassium phosphate, 1 mM EDTA, and 1 mM β-mercaptoethanol. After addition of phenylmethylsulfonyl fluoride (Sigma) to a final concentration of 0.5 mM, cells were disrupted by two passages through a French pressure cell at 18,000 lb/in^2 and centrifuged at 100,000 × *g* for 30 min at 4°C. Urease was resolved from cell extracts by chromatography on DEAE-Sepharose (30 by 2.5 cm), phenyl-Sepharose (30 by 2.5 cm), and Mono Q (HR 10/10) columns (Pharmacia, Piscataway, N.J.) as described previously (23).

Purification of UreE. Wild-type and H144* UreE proteins were purified by use of a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen, Chatsworth, Calif.) by modifications of the method described by Sriwanthana et al. (22). Cultures (2 liters) of *E. coli* DH5α(pKAUEFG), *E. coli* HMS174 (DE3)(pETWT), or *E. coli* HMS174 (DE3)(pETH144*) were harvested by centrifugation, resuspended in buffer A (20 mM Tris [pH 7.9], 500 mM NaCl, 60 mM imidazole) containing 0.5 mM phenylmethylsulfonyl fluoride, and disrupted by two passages through a French pressure cell at 18,000 lb/in^2. Cell debris was removed by centrifugation at 100,000 × *g* for 40 min at 4°C. The Ni-NTA resin was charged with 50 mM $NiSO_4$ and equilibrated with buffer A. Cell extracts were loaded onto the column and washed with buffer A until the A_{280} reached baseline. Bound proteins were eluted in 20 mM Tris (pH 7.9)–500 mM NaCl–1 M imidazole. Fractions were analyzed by gel electrophoresis (see below), and samples of interest were dialyzed against 50 mM Tris (pH 7.9)–1 mM EDTA. Sample concentrations were adjusted by dilution or by concentration in a Centriprep-10 (Amicon, Beverly, Mass.).

Polyacrylamide gel electrophoresis. All denaturing gel electrophoresis was carried out with buffers as described by Laemmli (8) and with a 12% polyacrylamide resolving gel with a 4.5% polyacrylamide stacking gel. Samples were denatured at 100°C for 5 min in buffer containing 3% sodium dodecyl sulfate and 5% β-mercaptoethanol. Native gel electrophoresis utilized the same buffers without detergent and consisted of a 3% polyacrylamide stacking gel and a 7.5% polyacrylamide resolving gel. Gels were stained with Coomassie brilliant blue, or in the case of selected denaturing gels, proteins were blotted onto Immobilon P nylon membranes (Millipore, Bedford, Mass.), probed with anti-UreE antibodies (10), and detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (1).

N-terminal sequence analysis. Purified H144* UreE (200 pmol) was resolved on a denaturing 12% polyacrylamide gel and electroblotted onto a Pro-Blot membrane (Applied Biosystems, Foster City, Calif.) with a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The bands were visualized by Coomassie blue staining, excised from the membrane, and analyzed with an Applied Biosystems model 477A automated sequencer in the Michigan State University Macromolecular Structural Facility.

Size exclusion chromatography. The native molecular weights for wild-type and H144* UreE proteins were estimated by use of Superose 12 column chromatography (48.5 by 1.6 cm; Pharmacia). Isocratic elution utilized a mixture containing 50 mM Tris (pH 7.6), 1 mM EDTA, 1 mM β-mercaptoethanol, and 200 mM NaCl. The column was standardized with thyroglobulin, gamma globulin, ovalbumin, carbonic anhydrase, myoglobin, and vitamin B_{12} (M_r = 670,000, 158,000, 44,000, 29,000, 17,000, and 1,350, respectively).

Equilibrium dialysis. Protein samples (5 μM peptide in 0.5 ml) were dialyzed against $^{63}NiCl_2$ (1,445 mCi/mmol; Du Pont NEN Research Products, Inc., Wilmington, Del.) diluted with various concentrations of unlabelled $NiCl_2$ in 0.5 ml with an equilibrium microvolume dialyzer (Hoefer Scientific Products, San Francisco, Calif.) equipped with precut dialysis membranes (molecular weight cutoff, 12,000 to 14,000). The buffers used were either 50 mM sodium phosphate (pH 7.2) or 50 mM Tris (pH 7.6), each containing 85 mM NaCl, and in selected experiments, additional metal ions were present as indicated. After overnight equilibration at room temperature, an aliquot from each compartment was measured for radioactivity with a Beckman LS7000 liquid scintillation system and Safety Solve scintillation fluid (Research Products International Corporation, Mount Prospect, Ill.). The data were fitted by the method of least squares to the following equation for fractional saturation (Y):

$$Y = \frac{[\mathrm{Ni}]\{1/K_1 + ([\mathrm{Ni}]/K_1K_2) + ([\mathrm{M}]/K_1K_4) + ([\mathrm{M}]/K_2K_3)\}}{1 + (2[\mathrm{Ni}]/K_1) + ([\mathrm{Ni}]^2/K_1K_2) + (2[\mathrm{M}]/K_3) + ([\mathrm{M}]^2/K_3K_4) + (2[\mathrm{Ni}][\mathrm{M}]/K_1K_4) + (2[\mathrm{Ni}][\mathrm{M}]/K_2K_3)}$$

where K_1 and K_2 are dissociation constants for binding the first and second nickel ions, M is the competing divalent metal ion, and K_3 and K_4 are the dissociation constants for binding the first and second competing divalent metal ions (the dissociation constant for nickel ion binding to protein with one other metal bound is assumed to be equal to K_2, and the dissociation constant for binding of a metal to protein with one nickel bound is assumed to be equal to K_4).

UV-visible spectroscopy. Electronic absorption spectra of H144* UreE protein (40 μM) in the presence of various divalent metal ion concentrations were recorded and analyzed on a DU-7500 UV-visible spectrophotometer (Beckman, Fullerton, Calif.) by use of a 150-μl cuvette and 50 mM Tris–85 mM NaCl (pH 7.6) buffer. The data for fitting the absorbance changes with copper ion binding were derived with the following equation for fraction absorbance (Y_A):

$$Y_A = \frac{[\mathrm{Cu}]^2/(K_1K_2)}{1 + 2[\mathrm{Cu}]/K_1 + [\mathrm{Cu}]^2/K_1K_2}$$

RESULTS

Functional analysis of the UreE His-rich region. A site-directed mutation was generated and urease activity assays were used to examine whether the UreE His-rich carboxyl terminus participates in urease metallocenter assembly via its ability to specifically bind nickel ions. The *ureE* gene in pKAU17, containing the intact *K. aerogenes ureDABCEFG* urease gene cluster, was mutated to place a stop codon at the position normally encoding His-144. The resulting H144* UreE peptide lacks the carboxyl-terminal 15 residues, including 10 histidines that were proposed to be involved in metal binding (10). The mutated plasmid pKAU17H144*, pKAU17,

TABLE 1. Specific activities of urease purified from *E. coli* DH5α or *E. coli* C *slyD* containing the *K. aerogenes* urease gene cluster with UreE variants

Host	Plasmid derivative	Sp act	
		U/mg	%
E. coli DH5α	pKAU17	1,778 ± 72	100
	pKAU17H144*	1,300 ± 75	73
	pKAU17Δ*ureE-1*	1,068 ± 21	60
	pKAU17Δ*ureE-2*	1,128 ± 55	63
E. coli C *slyD*	pKAU17	1,730 ± 75	100
	pKAU17H144*	1,350 ± 80	78
	pKAU17Δ*ureE-1*	1,097 ± 47	63
	pKAU17Δ*ureE-2*	1,152 ± 30	67

and two *ureE* deletion derivatives (pKAU17Δ*ureE-1* and pKAU17Δ*ureE-2*) (9) were transformed into *E. coli* DH5α and *E. coli* C *slyD* cells for analysis of urease activity effects. The latter host was used to eliminate possible compensatory metal-binding interactions by SlyD (containing a His-rich motif with 14 histidines in a stretch of 31 residues) that was absent in this strain (20) but presumed to be present in *E. coli* DH5α. As shown by representative data in Table 1, the absence of the UreE carboxyl-terminal region resulted in a decrease of approximately 25% in the specific activity of urease purified from DH5α or *slyD* cells grown in LB medium containing 1 mM $NiCl_2$ when compared with the activity of control cells synthesizing wild-type UreE. Enzymes isolated from cells containing *ureE* deletion derivatives were further depressed in activity. It should be noted that the observed activities for urease obtained from *E. coli* DH5α(pKAU17), *E. coli* DH5α(pKAU17 Δ*ureE-1*), and *E. coli* DH5α(pKAU17Δ*ureE-2*) were somewhat elevated compared with previously reported values (1,487, 966, and 708 U/mg, respectively [9]). As noted earlier (9), the specific activity for urease obtained from *E. coli* cells is depressed compared with that for enzyme isolated from *K. aerogenes* (~2,500 U/mg).

In an extension of the studies described above, the effect of each *ureE* deletion on functional urease metallocenter assembly was examined in cell extracts of strains grown in LB and MOPS-glutamine media containing varied concentrations of $NiCl_2$ (Fig. 1). Urease specific activities in cell extracts of pKAU17H144* closely paralleled those for *E. coli* DH5α cells containing pKAU17 at all nickel ion concentrations but were somewhat lower, especially in the LB medium. In contrast, the two deletion mutants showed a more dramatic diminishment in specific activity at all nickel concentrations in both rich and minimal media. The ratios of activities observed for the 1 mM nickel ion concentration are in good agreement with the ratios seen for the purified enzymes (Table 1). These results indicate that while the lack of the His-rich carboxyl terminus of UreE had only a small effect on the extent of urease activation, the complete absence of UreE had a more marked effect.

Because UreE is synthesized at very high levels in cells containing the *K. aerogenes* urease gene cluster (9), we considered an alternative role for the carboxyl-terminal region of UreE; i.e., protection against nickel ion toxicity. To test whether the UreE His-rich motif functions in a cellular nickel ion resistance mechanism, the growth of cells harboring pKAU17 or pKAUH144* was examined with $NiCl_2$ concentrations of 0 to 3 mM. *E. coli* DH5α and *E. coli* C *slyD* cells carrying either of the plasmids mentioned above were inoculated into LB or MOPS-glutamine medium, and their growth rates were monitored. No consistent differences in growth rates were observed for these host-plasmid combinations in either medium at any nickel ion concentration (data not shown).

Purification of truncated UreE. Because the metal binding sites associated with the His-rich carboxyl terminus of UreE apparently were only partly involved in UreE-facilitated urease activation, we wanted to purify and characterize the truncated UreE protein to better define its role in the urease metallocenter assembly process. The method of Sriwanthana et al. (22) was used to purify wild-type *K. aerogenes* UreE from cell extracts of *E. coli* DH5α(pKAUEFG) or *E. coli* HMS174 (DE3)(pETWT) by Ni-NTA column chromatography, with yields of 5 to 20 mg of protein per liter of culture. Surprisingly, the truncated UreE in cell extracts of *E. coli* HMS174 (DE3)(pETH144*) was also able to be purified by this method, yielding 5 to 20 mg of H144* UreE per liter of culture. Even when an imidazole gradient was used during Ni-NTA chromatography, the H144* and wild-type UreE proteins were unable to be resolved because of their broad and overlapping elution profiles. The H144* UreE protein was estimated to have an M_r of ~36,700 on the basis of its elution position during gel exclusion chromatography. For comparison, similarly treated UreE yielded a native M_r value of ~37,600. As illustrated by the denaturing polyacrylamide gel shown in Fig. 2, a nearly homogeneous peptide was obtained. Consistent with a difference of 15 amino acid residues between this peptide and wild-type UreE, the apparent M_r values for the indicated bands were estimated to be 21,000 and 23,000, respectively. As noted

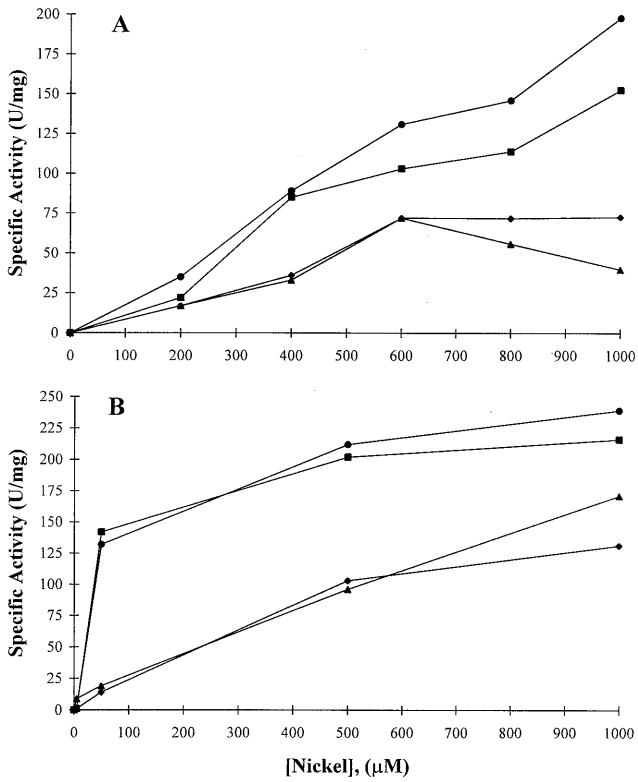

FIG. 1. Effect of nickel ion concentration in the medium on the level of urease specific activity in cell extracts. *E. coli* DH5 cells containing the wild-type urease gene cluster on plasmid pKAU17 (●), the urease gene cluster encoding the truncated UreE on pKAU17H144* (■), or *ureE* deletion plasmids pKAU17Δ*ureE-1* (▲) and pKAU17Δ*ureE-2* (◆) were cultured overnight in LB medium (A) or for 72 h in MOPS medium (B) in the presence of various nickel ion concentrations. The cell extracts were assayed for urease specific activity.

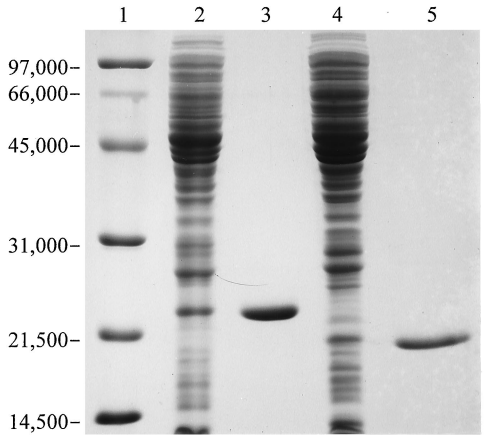

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic comparison of wild-type and truncated UreE peptides. Lanes: 1, molecular weight markers; 2, cell extract of *E. coli* HMS174 (DE3)(pETWT); 3, Ni-NTA pool of wild-type UreE; 4, cell extract of *E. coli* HMS174 (DE3)(pETH144*); 5, Ni-NTA pool of H144* UreE.

previously (9), UreE migrates as an anomalously large peptide compared with its size (M_r = 17,558) as determined by DNA sequence analysis (16), and this feature was also seen with the truncated peptide. N-terminal sequencing of the putative H144* peptide revealed a sequence (MLYLTQRLEI) that matched the predicted UreE sequence. In addition, the purified protein cross-reacted with anti-*K. aerogenes* UreE antibodies when examined by Western blot (immunoblot) analysis (data not shown).

Interaction of truncated UreE with metal ions. The truncated form of UreE was able to bind to a Ni-NTA column despite the absence of the His-rich region, consistent with a capacity of truncated UreE to bind metal ions. We examined the metal binding properties of truncated UreE protein by equilibrium dialysis and UV-visible spectroscopic analyses.

The number of nickel ions that bound to H144* UreE protein was examined for a range of nickel ion concentrations by equilibrium dialysis methods (Fig. 3). The data could not to be fitted to a simple model that assumed the presence of independent sites with identical binding affinities for this metal ion. Rather, the data suggested the presence of cooperativity in binding with approximately two nickel ions bound per dimer of truncated UreE, with binding constants of K_1 of 85 ± 10 μM and K_2 of 0.78 ± 0.1 μM for the first and second nickel ions, respectively. Half saturation was observed at ~8 μM nickel ion.

The specificity of the truncated UreE protein for binding nickel ions was analyzed by equilibrium dialysis in the presence of competing metal ions, as illustrated in Fig. 4. In these studies, the nickel ion concentrations were held constant at 10 μM, and the concentrations of several other divalent metal ions were varied. Magnesium, manganese, and calcium had no effect on nickel ion binding even when present at 400 μM concentrations. Cadmium and cobalt ions appeared to compete reasonably well for approximately half of the nickel ion binding subsites (K_3 = 55 ± 40 μM and K_4 = 35 ± 5 μM for cadmium; K_3 = 6 ± 2 μM and K_4 = 7 ± 1 μM for cobalt). Zinc and copper ions appeared to compete for nearly all of the nickel binding sites in the truncated protein, with K_3 equal to 25 ± 15 μM and K_4 equal to 2.0 ± 0.2 μM for zinc and K_3 equal to 23 ± 3 μM and K_4 equal to 0.10 ± 0.015 μM for copper.

The interaction between copper ions and H144* UreE protein was further characterized by UV-visible spectroscopy. As shown in Fig. 5A, the addition of copper ions to the truncated UreE protein led to the formation of an intense absorption band at ~365 nm and an additional broad peak at ~645 nm. Difference spectra were generated, and the effects of copper ion concentration on the ΔA_{365} values were measured (Fig. 5B). K_1 and K_2 values were estimated at 10,000 and 17 μM, respectively, with an approximate half saturation of copper ion

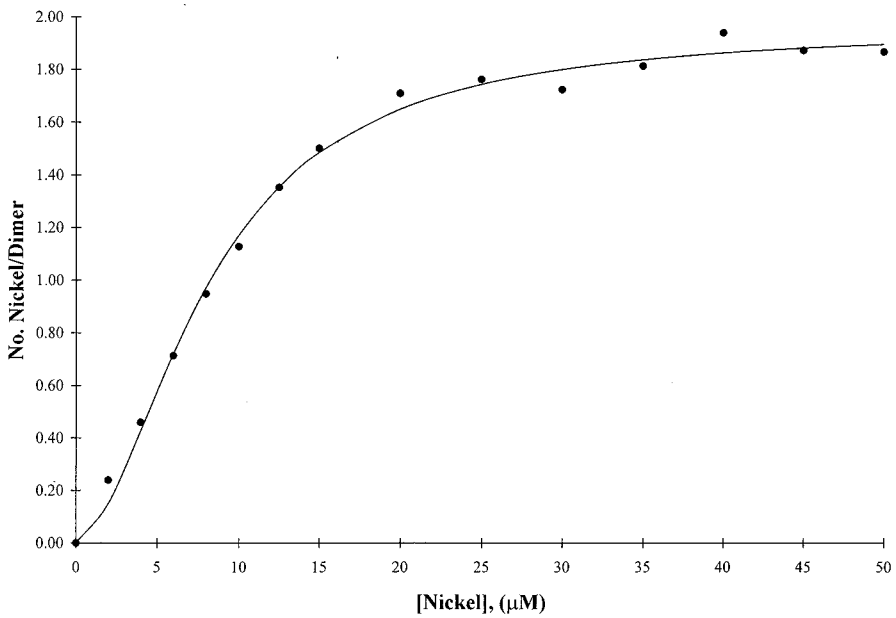

FIG. 3. Nickel ion binding by truncated UreE peptide based on equilibrium dialysis analysis. H144* UreE protein at a 5 μM dimer concentration in 50 mM sodium phosphate buffer (pH 7.2) containing 85 mM NaCl, was equilibrated with the indicated concentrations of $NiCl_2$, and the number of nickel ions bound per dimer was assessed. The data were fitted as described in Materials and Methods, with K_1 and K_2 equal to 85 and 0.78 μM, respectively.

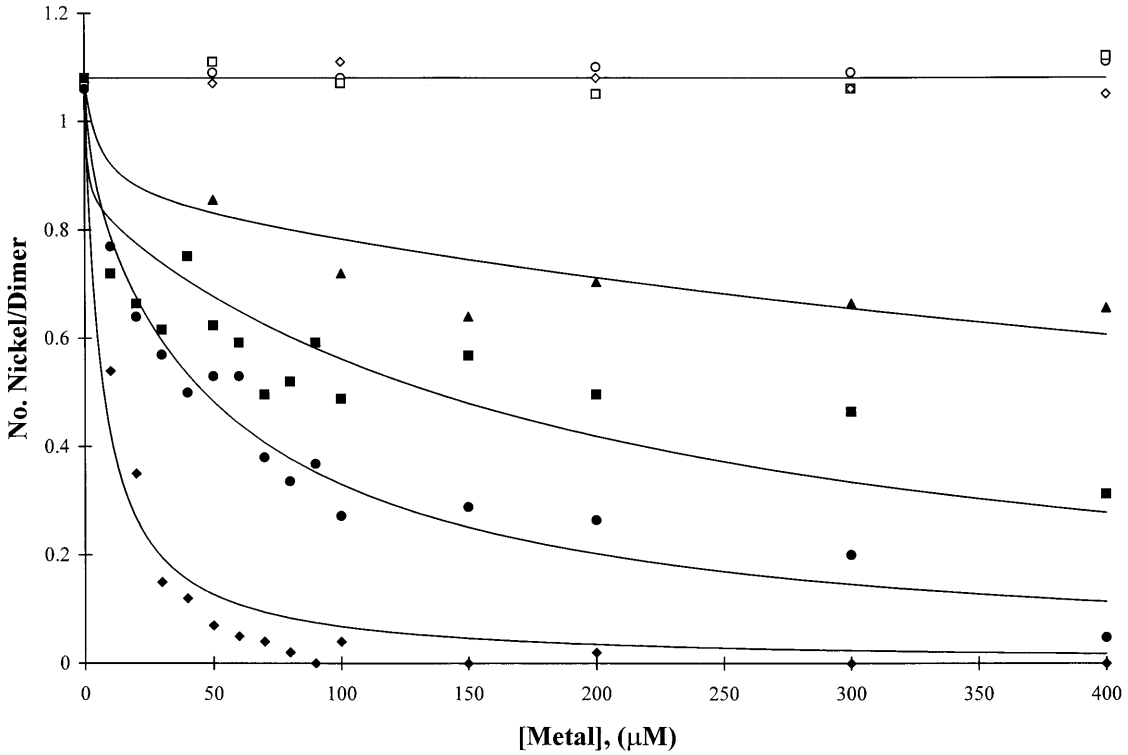

FIG. 4. Metal ion competition for nickel ion binding to truncated UreE peptide. Equilibrium dialysis of 5 μM H144* UreE peptide with nickel ion at a concentration of 10 μM was performed in the presence of various concentrations of divalent metal ions. Experiments with $MnCl_2$ (○), $MgCl_2$ (◇), and $CaCl_2$ (□) were carried out with 50 mM sodium phosphate buffer (pH 7.2)–85 mM NaCl. Experiments with $CdCl_2$ (▲), $CoCl_2$ (■), $ZnCl_2$ (●), and $CuCl_2$ (◆) were carried out with 50 mM Tris buffer (pH 7.6)–85 mM NaCl. The data were fitted as described in the text, with K_3 and K_4 values equal to 55 and 35 μM, respectively, for cadmium, 6 and 7 μM, respectively, for cobalt, 25 and 2 μM, respectively for zinc, and 23 and 0.1 μM, respectively, for copper.

binding to H144* UreE observed at 300 μM Cu(II). These data were unable to be fitted reasonably when assuming that the absorbance was derived from all copper ions that bound to the protein. Rather, the data were fitted well when the assumption was made that only the species with two copper ions bound gave rise to this spectrum. In contrast to the copper-dependent change, no or only very small absorbance changes were noted when the truncated sample was titrated with nickel, cobalt, or manganese ions. Upon addition of elevated levels of zinc or cadmium (>300 μM), the protein solution was observed to turn cloudy, consistent with denaturation.

DISCUSSION

Role of the His-rich carboxyl terminus of *K. aerogenes* UreE. We have obtained evidence that for the most part negates the hypothesis that the carboxyl-terminal His-rich domain of the UreE protein facilitates *K. aerogenes* urease metallocenter assembly. This hypothesis was based on (i) studies demonstrating an involvement of UreE in urease metallocenter assembly (9), (ii) the ability of UreE to bind nickel (10), (iii) the UreE sequence that revealed a His-rich motif (16), and (iv) the known ability of His-rich sequences to bind nickel ions (6). We observed, however, that urease specific activities in cells that produce UreE lacking the His-rich region are only moderately depressed when compared with that of cells containing wild-type UreE. In contrast to the small effects seen for cells possessing truncated UreE, *ureE* deletion mutants (possessing no cross-reactive material when tested with anti-*K. aerogenes* UreE antibodies) exhibit greater reductions in urease activity. These results confirm previous reports in *K. aerogenes* (9) and agree with studies of deletion mutants in *Proteus mirabilis* (22) showing that the presence of UreE facilitates urease activation.

Because the carboxyl terminus of UreE plays, at most, a limited role in urease metallocenter assembly, it is reasonable to suggest that the His-rich region possesses an alternative function. We examined the possibility that this region may confer enhanced resistance to nickel ion toxicity; however, studies comparing the levels of growth of cells producing wild-type and truncated forms of UreE in the presence of elevated nickel ion concentrations fail to provide evidence in support of this role. Another potential function of this region is to serve a nickel storage role. For example, during growth in medium with abundant nickel ions, the metal may be bound to the His-rich region, and this metal ion sink could perhaps be utilized if growth conditions changed to one of nickel deficiency. Although we have no experimental evidence specific to *K. aerogenes* related to this point, comparative UreE sequence analyses may be relevant. Whereas His-rich regions are known or predicted to be present in UreE peptides from *K. aerogenes* (16), *P. mirabilis* (7), *Yersinia enterocolitica* (4), and *Haemophilus influenza* (5), the peptides from *Helicobacter pylori* (3), *Bacillus* sp. strain TB-90 (13), *Bacillus pasteurii* (25), *Streptococcus salivarius* (2), and *Ureaplasma urealyticum* (18) do not possess this motif. In two of these microbes, nickel ion-specific permeases are proposed to be present, i.e., UreH in *Bacillus* sp. strain TB-90 (13) and NixA in *Helicobacter pylori* (13a). Furthermore, the *S. salivarius* urease gene cluster appears to encode a membrane-associated protein that may be related to these permeases (2). An intriguing hypothesis requiring further study is that cells possessing a nickel-specific permease do

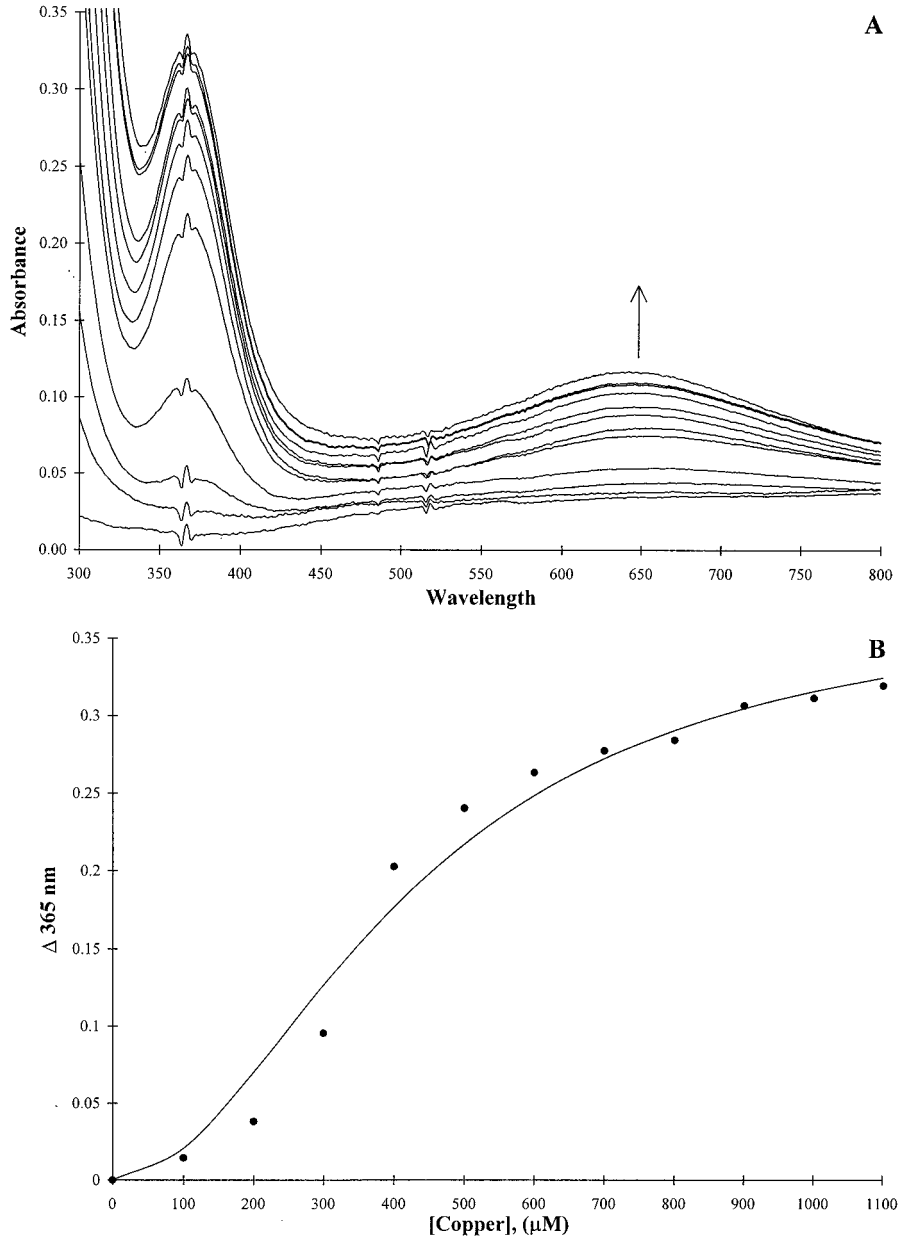

FIG. 5. UV-visible spectroscopic analysis of the interactions between copper ions and truncated UreE protein. (A) The protein (40 μM) was titrated with 100 μM increments of $CuCl_2$ in 50 mM Tris (pH 7.6) buffer containing 85 mM NaCl, and the spectra were corrected for dilution effects. The arrow indicates increasing copper ion concentration. (B) Effect of copper ion concentrations on the A_{365} difference for truncated UreE (●). The data were fitted as described in the text, with K_1 and K_2 equal to 10,000 and 17 μM, respectively.

not require, and typically lack, a His-rich domain on UreE, whereas cells that do not possess an efficient uptake system utilize the UreE His-rich domain to store nickel ions until required.

Properties and function of truncated UreE. Because the truncated UreE peptide is able to facilitate urease activation, we purified and characterized several properties of this protein. Similar to the native UreE protein, the H144* protein appears to be dimeric. Furthermore, despite loss of the His-rich motif, the truncated protein binds to a nickel-affinity column with the same conditions as those developed for purifying the wild-type protein. Consistent with the ability for H144* UreE to bind to a Ni-NTA column, nickel binding was directly demonstrated by equilibrium dialysis experiments. Because nickel ions bind to truncated UreE in a clearly cooperative manner, we reevaluated published data for the native protein that had been fitted with the assumption of independent sites (10). Our reanalysis reveals that the fit to the prior data can be improved by allowing for cooperativity, with approximately half the nickel ions binding with a K_1 of 85 μM and the rest binding with a K_2 of 0.5 μM and a maximum of about five nickel ions per dimer. For the native protein, competitive equilibrium dialysis experiments had revealed that while calcium, magnesium, and manganese ions are ineffective at competing with nickel ions, cobalt ions compete weakly for a portion of the sites, zinc ions are highly effective at competing for approx-

imately half of the total nickel binding sites, and copper ions compete weakly for all of the binding sites (10). Nickel ion binding to truncated UreE is similarly unaffected by calcium, magnesium, and manganese ions, but H144* UreE interactions with the other metals are significantly altered compared with that of the native protein. Cobalt and zinc ions (at 50 to 100 μM concentrations appear to compete well for approximately half of the nickel ion binding sites, and both ions can compete at higher concentrations for the second binding site in H144* protein. The most notable finding from the competitive equilibrium dialysis experiment is the high affinity of copper ions for the nickel binding site in the truncated protein. We attempted to use copper ions as a spectroscopic probe of the metal site, and electronic spectroscopy revealed a spectrum suggestive of an octahedral or trigonal-bipyramidyl Cu(II) geometry (11). This spectrum appears to be generated only upon binding of a second copper ion to the protein. We propose that the nickel ions bound to truncated UreE are related to the role of this protein in urease activation and that the copper-substituted protein may be a useful species for characterization of the metal binding site.

ACKNOWLEDGMENTS

We thank Ry Young for providing *E. coli* C *slyD* cells and members of the laboratory for critical review of the manuscript.

This work was supported by the National Institutes of Diabetes and Digestive and Kidney Diseases (DK 45686) and by the Michigan State University Agricultural Experiment Station.

REFERENCES

1. **Blake, M. S., K. H. Johnston, G. L. Russel-Jones, and E. C. Gotschlich.** 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. **136:**175–179.
2. **Chen, Y.-Y. M., K. A. Clancy, and R. A. Burne.** 1996. *Streptococcus salivarius* urease: genetic and biochemical characterization and expression in a dental plaque streptococcus. Infect. Immun. **64:**585–592.
3. **Cussac, V., R. L. Ferrero, and A. Labigne.** 1992. Expression of *Helicobacter pylori* urease genes in *Escherichia coli* grown under nitrogen-limiting conditions. J. Bacteriol. **174:**2466–2473.
4. **de Koning-Ward, T. F., A. C. Ward, and R. M. Robins-Browne.** 1994. Characterization of the urease-encoding gene complex of *Yersinia enterocolitica*. Gene **145:**25–32.
5. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J.-F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L.-I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
6. **Hochuli, E., W. Bannwarth, H. Döbeli, R. Gentz, and D. Stüber.** 1988. Genetic approach to facilitate purification of recombinant proteins with a novel chelate adsorbent. Bio/Technology **Nov.:**1321–1325.
7. **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.
8. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
9. **Lee, M. H., S. B. Mulrooney, M. J. Renner, Y. Markowicz, and R. P. Hausinger.** 1992. Sequence of *ureD* and demonstration that four accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) are involved in nickel metallocenter biosynthesis. J. Bacteriol. **174:**4324–4330.
10. **Lee, M. H., H. S. Pankratz, S. Wang, R. A. Scott, M. G. Finnegan, M. K. Johnson, J. A. Ippolito, D. W. Christianson, and R. P. Hausinger.** 1993. Purification and characterization of *Klebsiella aerogenes* UreE protein: a nickel-binding protein that functions in urease metallocenter assembly. Protein Sci. **2:**1042–1052.
11. **Lever, A. B. P.** 1984. Inorganic electronic spectroscopy, 2nd ed. Elsevier, New York.
12. **Lowry, O. H., N. J. Rosebrough, A. J. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
13. **Maeda, M., M. Hidaka, A. Nakamura, H. Masaki, and T. Uozumi.** 1994. Cloning, sequencing, and expression of thermophilic *Bacillus* strain TB-90 urease gene complex in *Escherichia coli*. J. Bacteriol. **176:**432–442.

13a. **Mobley, H. L. T., R. M. Garner, and P. Baverfeind.** 1995. *Helicobacter pylori* nickel-transport gene *nixA*: synthesis of catalytically active urease in *Escherichia coli* independent of growth conditions. Mol. Microbiol. **16:**97–109.

14. **Mobley, H. L. T., M. D. Island, and R. P. Hausinger.** 1995. Molecular biology of microbial ureases. Microbiol. Rev. **59:**451–480.
15. **Moncrief, M. B. C., and R. P. Hausinger.** 1996. Nickel incorporation into urease, p. 151–171. *In* R. P. Hausinger, G. L. Eichhorn, and L. G. Marzilli (eds.), Mechanisms of metallocenter assembly. VCH Publishers, New York.
16. **Mulrooney, S. B., and R. P. Hausinger.** 1990. Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. J. Bacteriol. **172:**5837–5843.
17. **Mulrooney, S. B., H. S. Pankratz, and R. P. Hausinger.** 1989. Regulation of gene expression and cellular localization of cloned *Klebsiella aerogenes* (*K. pneumoniae*) urease. J. Gen. Microbiol. **135:**1769–1776.
18. **Neyrolles, O., S. Ferris, N. Behbahani, L. Montagnier, and A. Blanchard.** 1996. Organization of *Ureaplasma urealyticum* urease gene cluster and expression in a suppressor strain of *Escherichia coli*. J. Bacteriol. **178:**647–655.
19. **Park, I.-S.** 1994. Ph.D. thesis. Michigan State University, East Lansing.
20. **Roof, W. D., S. M. Horne, K. D. Young, and R. Young.** 1994. *slyD*, a host gene required for ΦX174 lysis, is related to the FK506-binding protein family of peptidyl-prolyl *cis-trans*-isomerases. J. Biol. Chem. **269:**2902–2910.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Sriwanthana, B., M. D. Island, D. Maneval, and H. L. T. Mobley.** 1994. Single-step purification of *Proteus mirabilis* urease accessory protein UreE, a protein with a naturally occurring histidine tail, by nickel chelate affinity chromatography. J. Bacteriol. **176:**6836–6841.
23. **Todd, M. J., and R. P. Hausinger.** 1989. Competitive inhibitors of *Klebsiella aerogenes* urease. Mechanisms of interaction with the nickel active site. J. Biol. Chem. **264:**15835–15842.
24. **Weatherburn, M. W.** 1967. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. **39:**971–974.
25. **You, J.-H., B.-H. Song, J.-G. Kim, M.-H. Lee, and S.-D. Kim.** 1995. Genetic organization and nucleotide sequencing of the *ure* gene cluster in *Bacillus pasteurii*. Mol. Cells **5:**359–369.