Cloning of Urease Gene Sequences from *Providencia stuartii*

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Providencia stuartii was the most prevalent isolate recovered from urine specimens taken weekly over a 1-year period from 51 nursing home patients with urinary catheters in place. Thirty percent of the isolates were urease positive. Urease, which is implicated in renal stone formation, was shown to be transmissible on an 82-kilobase conjugative plasmid in one isolate. Plasmid DNA isolated from this strain was digested with *EcoRI*, ligated into the *EcoRI* site of pBR322, and used to transform *Escherichia coli* HB101. Ampicillin-resistant clones were replica plated onto urea segregation agar, and a urease-positive clone, designated pMID101, was isolated. Recombinant and native urease from cell lysates had identical electrophoretic mobilities on nondenaturing polyacrylamide urease activity gels. The native enzyme was induced fourfold when cells were grown in the presence of 0.1% urea and had a K_m of 9.4 mM and a V_{max} of 3.2 μmol of NH_3 per min per mg of protein. Its molecular weight was estimated to be $375,000 \pm 35,000$ by Sephacryl S-300 chromatography. The enzyme was cytoplasmic in *P. stuartii*, was inhibited in vitro by hydroxyurea, acetohydroxamic acid, and EDTA, and appears to have a complex subunit structure and a unique molecular size within genera of the *Proteeae* tribe.

Urinary incontinence in nursing home patients is often managed with an indwelling urinary catheter. Long-term catheterization is universally associated with bacteriuria (37). In a recent study by our group, nearly half of the isolates cultured from urine specimens taken weekly from 51 chronically catheterized patients produced the enzyme urease (unpublished observation). The most prevalent isolate in this study was *Providencia stuartii* (37), which has been shown to encode urease on a conjugative plasmid (10, 24).

Urease (urea amidohydrolase EC 3.5.1.5) is found in over 200 species of bacteria (5, 31). The enzyme from bacterial and plant sources has been reported to have a high molecular weight (200,000 to 800,000) (7, 9, 30, 33; J. A. Anderson, F. Kopko, A. J. Siedler, and C. G. Nohle, *Fed. Proc.*, p. 767, 1969) and contains nickel ions in the active site (2, 6, 33). Jack bean urease is composed of six identical subunits (7). The enzyme catalyzes the hydrolysis of urea [$\text{CO}(\text{NH}_2)_2$] to yield CO_2 and NH_3 and has been implicated in the cellular regulation of nitrogen metabolism (9, 16). The bacterial enzyme is cytoplasmic (9, 27) and demonstrates high specificity for its substrate, recognizing only six other analogs, *N*-hydroxyurea, *N,N'*-dihydroxyurea, *N*-aminourea, *N*-methylurea, formamide, and acetamide. The result of urea hydrolysis is a rapid rise in the pH of the reaction mixture due to release of the ammonium ion. This enzyme reaction is of special importance in the urinary tract because of the high substrate concentration (urea concentration in urine, 25 to 30 mg/ml or 0.4 to 0.5 M [11]).

Bacterial urease is recognized as a virulence factor principally because of its role in kidney and bladder stone formation (4, 11, 20, 26, 31, 35, 39). Alkalinization of the urine by urease-catalyzed urea hydrolysis facilitates the precipitation of polyvalent cations and anions. Struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) and carbonate-apatite, $[\text{Ca}_{10}(\text{PO}_4)_6\text{CO}_3(\text{OH})_2]$ are the principal in vivo or in vitro insolubility products in urine with a pH of 8.5 or greater (11). The proportion of all urinary stones due to infection has been estimated to be between 20 and 40% (11) and is particularly

high in chronically catheterized patients with spinal cord injury (26). Indeed, electron micrographs of stone cross sections have demonstrated bacteria as the seeds for crystal formation (36).

Although jack bean urease has been studied extensively (e.g., see references 2, 6, and 7), only a limited number of studies have characterized the ureases of the common bacterial isolates from urinary tract infection. Senior et al. (34) demonstrated that representatives of the *Proteeae* tribe (*Proteus vulgaris*, *Proteus mirabilis*, *Proteus morganii*, and *Proteus rettgeri*) produced ureases of different electrophoretic mobilities. Rosenstein et al. (30) also found that the molecular weights of ureases of the *Proteeae* differed and that *P. morganii* (now *Morganella morganii*) was more resistant to two urease inhibitors. Guo and Liu (12) found that the *P. morganii* urease was serologically distinct from that of *P. mirabilis*, *P. vulgaris*, and *P. rettgeri* (now *Providencia rettgeri*). Because the enzymes from different bacterial species seem to possess different properties and because the few existing studies do not convincingly resolve the role of *Proteeae* urease in pathogenicity, there is a need for careful characterization of these enzymes and the mechanisms by which they can be effectively inhibited.

This report describes the cloning of the gene sequences encoding the urease from a conjugative plasmid isolated from a urease-positive isolate of *P. stuartii* and characterization of the gene product.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. stuartii* BE2467 was isolated from the urine of a nursing home patient with a urinary catheter in place (24). The organism was identified from a pure culture with the Minitek Enterobacteriaceae II system (BBL Microbiology Systems, Cockeysville, Md.) and differentiated from closely related species by fermentation of adonitol (BBL), inositol (BBL), galactose (J. T. Baker Chemical Co., Phillipsburg, N.J.), and trehalose (Eastman Kodak Co., Rochester, N.Y.) (14).

Escherichia coli HB101 (*pro leu rpsL recA lacYI*) was used as the recipient for transformation with recombinant

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FIG. 1. Deletion analysis and transposon mutagenesis of recombinant urease-encoding plasmids. An 82-kb conjugative plasmid from urease-positive *P. stuartii* BE2467 and vector pBR322 were digested to completion with *Eco*RI (E), ligated, and used to transform *E. coli* HB101. Ampicillin-resistant transformants were screened for urease production on urea segregation agar. Plasmid DNA from a urease-positive clone, designated pMID101, was isolated on a cesium chloride gradient and digested with various restriction endonucleases. The plasmid represents a 17-kb *Eco*RI fragment derived from *P. stuartii* and cloned into the *Eco*RI site of pBR322 (thickened lines). The restriction sites were determined by estimating the fragment sizes derived from single, double, and triple digests on ethidium bromide-stained agarose gels. Various deletion mutants of pMID101 and pMID201 were generated by digesting them with either *Pst*I (P) or *Ava*I (A) and religating. The *Hind*III site (H) is also indicated. Urease activity is noted beside each plasmid (+, positive; -, negative). Plasmid pMID204 represents an *Ava*I deletion of pMID201 and encodes a functional urease. Restriction endonuclease recognition sites are shown below the line. The thickened lines represent the remaining portion of vector pBR322. Ori, Origin of replication. The sites of insertion of the 3.3-kb *Hind*III fragment encoding kanamycin resistance into pMID201 are shown for reference (◆; Fig. 2). The sites of the lambda1105 transposon-insertions (▼) are also shown. The resulting plasmids were screened for urease activity. Plasmid designations (pMID) are shown above the insertion sites.

plasmids (21). *E. coli* P678-54 was used for minicell experiments.

Bacteria were maintained on L agar (22) plates and transferred weekly. Cultures were grown in L broth (22) at 37°C with aeration (200 rpm). Where indicated, urease-positive strains were induced with 0.1% (wt/vol) urea which was added after the medium was autoclaved.

Plasmid isolation. Plasmid DNA was isolated from cultures (200 ml) of *P. stuartii* and *E. coli* grown in L broth. Cells were harvested, washed, and lysed with alkaline sodium dodecyl sulfate (SDS) (3). DNA was purified by centrifugation to equilibrium in cesium chloride-ethidium bromide density gradients (21).

Construction of recombinant plasmids encoding urease. An 82-kilobase (kb) plasmid (5 µg of DNA) encoding urease (24), isolated from *P. stuartii* BE2467, and the vector plasmid pBR322 (0.2 µg of DNA) were digested to completion with *Eco*RI (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) in accordance with the specifications of the

manufacturer, ligated with T4 ligase (Bethesda Research) for 18 h at 15°C, and used to transform competent cells (19) of *E. coli* HB101. Recombinant clones were selected on L agar containing ampicillin (200 µg/ml) and screened by replica plating onto urea segregation agar (8). Urease-positive colonies were inoculated into broth, and plasmid DNA was isolated and digested with *Eco*RI. The fragments were sized by electrophoresis on 0.7% (wt/vol) agarose (Bethesda Research) gels with a Tris-borate-EDTA buffer system (21). Deletion mutations were constructed by digestion of the recombinant plasmids pMID101 and pMID201 (Fig. 1) with *Pst*I or *Ava*I (Bethesda Research) and religation. A map of the restriction endonuclease sites was determined by the relative sizes of the DNA fragments derived from digestions with one, two, or three enzymes (21).

Transposon mutagenesis. *E. coli* HB101(pMID204) was infected with lambda1105, which carries the kanamycin resistance gene of Tn903 flanked by Tn10 insertion sequences, by the method of Way et al. (38). Plasmid DNA was isolated from

kanamycin-resistant transformants and used to transform *E. coli* HB101. Kanamycin-resistant transformants were screened for urease activity by replica plating on urea segregation agar. The site and orientation of the transposed element in pMID204 were estimated by single digests of plasmid DNA with *Hind*III and *Ava*I.

Urease preparations. Cells were grown in L broth (100 ml) with and without urea and harvested by centrifugation at 4°C. The cells were washed three times with 20 mM sodium phosphate (pH 6.8). The cell pellet was suspended in 5 ml of 20 mM sodium phosphate (pH 6.8)–5 mM 2-mercaptoethanol–1 mM EDTA and ruptured in a precooled French pressure cell at 20,000 lb/in². The lysate was centrifuged at 27,000 × *g* for 30 min, and the supernatant was removed with a Pasteur pipette and used for all assays. Protein was determined by the method of Lowry et al. (19).

Spectrophotometric assay of urease. The alkalimetric assay of Hamilton-Miller and Gargan (13) was calibrated for the measurement of ammonia generated by urease-catalyzed urea hydrolysis. A cell lysate (2.0 ml, 14.4 mg of protein per ml) from *P. stuartii* BE2467, induced with urea and acetohydroxamic acid (29), was added to 98 ml of 4.5 mM sodium phosphate (pH 6.8) containing urea (2.3 mg/ml) at 23°C. At 1-min intervals, 2.75 ml was sampled and added to a 1-cm cuvette containing 0.25 ml of phenol red (80 µg/ml) solution, and the optical density at 560 nm (OD₅₆₀) was measured against a blank of the reaction solution containing no urea. Simultaneously, 24 µl of solution was added to an iced tube containing 0.39 ml of phenol nitroprusside. Ammonia was determined colorimetrically by the Berthelot reaction, with urea ammonia liberated by jack bean urease as a standard (34). The pH was monitored directly during the reaction with a combination electrode (Beckman Instruments, Inc., Fullerton, Calif.).

Fractionation of whole cells. Stationary cultures (1 liter) of *P. stuartii* BE2467 and *E. coli* HB101(pMID201) grown at 37°C with aeration (200 rpm) in Luria broth supplemented with 0.1% urea and with kanamycin (25 µg/ml) for *P. stuartii* or tetracycline (10 µg/ml) for *E. coli* were harvested by centrifugation. Samples of the supernatants were adjusted to pH 6.8 and placed on ice. The cell pellets were washed two times with 10 mM Tris hydrochloride (pH 8.0) and subjected to the cold osmotic shock procedure described by Rosen and Heppel (28). The shock fluid was adjusted to pH 6.8. The cell pellets were suspended in 20 ml of cold mM sodium phosphate (pH 6.8) and ruptured in a French pressure cell at 20,000 lb/in². The cell lysates were centrifuged at 10,000 × *g* for 10 min at 4°C to remove cellular debris, and the supernatant fluid was centrifuged at 100,000 × *g* for 60 min at 4°C. The supernatant (cytosol) was placed on ice. The pellet (membrane fraction) was washed two times in sodium phosphate buffer and suspended in a final volume of 3 ml of the same buffer. All fractions were assayed spectrophotometrically for urease activity. Protein was determined by the method of Lowry et al. (19). The activities of control enzymes were determined for each cell fraction. Lactate dehydrogenase activity was measured by the NADH-coupled spectrophotometric assay described by King (17). Beta-galactosidase activity was measured spectrophotometrically by monitoring the hydrolysis of *o*-nitrophenylgalactoside (32). NADH dehydrogenase activity was measured spectrophotometrically as described by Adler et al. (1). Alkaline phosphatase activity was determined by monitoring the hydrolysis of *p*-nitrophenylphosphate (25).

Nondenaturing gel electrophoresis. The urease activity of electrophoresed whole-cell soluble protein was visualized by

a modification of the method of Senior et al. (34). A slab 7% polyacrylamide resolving gel (15 by 15 by 0.15 cm; 1:32, bisacrylamide:acrylamide; U.S. Biochemical) in 380 mM Tris (pH 8.9)–1 mM EDTA–5 mM 2-mercaptoethanol with a 4% stacking gel in 63 mM Tris hydrochloride (pH 6.7) was loaded with cell lysate and electrophoresed with 380 mM glycine in 50 mM Tris as a running buffer at 60 V at 4°C for 16 h. The gel was removed from the apparatus and soaked in 10 to 15 changes (about 200 ml each) of 0.02% (wt/vol) cresol red–0.1% (wt/vol) EDTA until the gel remained yellow. The solution was drained well, and the gel was immersed in a 1.5% (wt/vol) urea solution. After the development of localized red bands, the gel was photographed on a light box with Kodachrome 64 slide film (Eastman Kodak). Jack bean urease (Sigma Chemical Co., St. Louis, Mo.) was used as an activity control.

Column chromatography. Cell lysate (15 mg of protein in 4 ml of 20 mM sodium phosphate–0.02% [wt/vol] sodium azide [pH 6.8]) derived from *P. stuartii* BE2467 grown in L broth supplemented with 1% (wt/vol) urea and acetohydroxamic acid (100 µg/ml) (29) was loaded onto a Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, N.J.) column (2.5 by 80 cm) and eluted at a flow rate of 30 ml/h. The urease activity for each fraction was measured by the spectrophotometric assay. The column was calibrated with blue dextran (2,000 kilodaltons [kDa]; Pharmacia) for void volume determination. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and bovine serum albumin (67 kDa) were used as molecular weight standards.

Minicell labeling and SDS-polyacrylamide gel electrophoresis. Minicells were isolated from *E. coli* P678-54 containing recombinant plasmids by differential and sucrose density centrifugations as previously described (23). The minicells were suspended to an OD₆₁₅ of 0.2 in M-9 minimal medium supplemented with glucose (0.5%) plus the 19 common amino acids (50 µg/ml), excluding methionine. To each tube (0.5 ml), 50 µCi of L-[³⁵S]methionine (600 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added (15). No carrier methionine was added. The tubes were incubated at 37°C for 60 min. The minicells were treated at 100°C for 5 min in SDS-gel sample buffer (18). Solubilized proteins (4 × 10⁵ trichloroacetic acid-precipitable cpm per lane) were electrophoresed on SDS-polyacrylamide (10% acrylamide, 1:32 bisacrylamide:acrylamide) by the method of Laemmli (18). After electrophoresis, gels were fixed, stained with Coomassie blue, dried onto filter paper, and autoradiographed with XAR-2 X-ray film (Eastman Kodak) for 18 h.

RESULTS

Cloning of genes encoding urease. An 82-kb conjugative plasmid, encoding urease, sucrose utilization, and resistance to kanamycin and mercury, isolated from *P. stuartii* BE2467 (24) was digested with *Eco*RI and ligated with *Eco*RI-digested pBR322. *E. coli* HB101, transformed with the ligation mixture, was selected for ampicillin resistance and screened for urease production on urea segregation agar. A urease-positive clone, designated pMID101, yielded a 17-kb *Eco*RI fragment from the *P. stuartii* plasmid and vector DNA when digested with *Eco*RI (Fig. 1). The 17-kb *Eco*RI fragment corresponded to one of the two largest restriction fragments of the conjugative plasmid isolated from *P. stuartii* BE2467.

A single deletion of a 4-kb *Pst*I fragment by digestion and religation yielded urease-positive, tetracycline-resistant,

FIG. 2. Insertional inactivation of urease gene. Plasmid pMID201 encoding urease was linearized with *Hind*III (H) by partial digestion and ligated with a 3.3-kb *Hind*III fragment encoding resistance to kanamycin from pHU859. Transformants were selected on L agar containing kanamycin and screened for urease activity (+, positive; -, negative). The site of insertion and orientation of the fragment were determined by digestion of plasmid DNA isolated from transformants with *Ava*I (Fig. 1). A, Two closely spaced *Ava*I sites within the 3.3-kb fragment.

ampicillin-sensitive pMID201. Deletion of one or two *Ava*I fragments from pMID101 to produce plasmids pMID202 and pMID203, respectively, resulted in loss of urease activity. However, the deletion of a 5.7-kb *Ava*I fragment from pMID201 resulted in a urease-positive clone, pMID204, which localized the urease genes to an 8.5-kb region.

Insertional inactivation. To further localize urease gene sequences, inactivation was attempted by insertion of the Tn5-derived kanamycin resistance gene into pMID201 (Fig. 2). Because no successful subclones were derived from experiments involving *Hind*III digestion, it was suspected that such a restriction site was within the urease-encoding gene. Plasmid pMID201 was linearized with *Hind*III and ligated to the 3.3-kb *Hind*III fragment encoding kanamycin resistance from pHU859 (the *Hind*III fragment from Tn5 inserted into the *Hind*III site of pBR322; obtained from J. Kaper, University of Maryland School of Medicine, Baltimore). Transformants were selected on kanamycin (50 μ g/ml) and screened for urease production. Plasmid DNA was isolated from both urease-positive and urease-negative transformants. Plasmids of approximately 20 kb were digested with *Ava*I to identify the site and orientation of the insertion.

Insertion of the *Hind*III fragment in either orientation into one of the four *Hind*III sites resulted in inactivation of urease (pMID304 and pMID305). When insertion was into a

FIG. 3. Induction of urease by urea. Cultures were grown in L broth containing various concentrations of urea at 37°C with aeration. The cells were harvested, washed in 20 mM sodium phosphate (pH 6.8), and ruptured in a French pressure cell, and insoluble material was removed by centrifugation. Urease activity was measured spectrophotometrically. Symbols: ▲, *E. coli*(pMID201); ●, *P. stuartii* BE2467.

*Hind*III site that was separated by 7.1 kb from the site of inactivation, the plasmid remained urease positive (pMID303).

To examine the possible subunit structure of urease and to look for the loss or truncation of a subunit polypeptide, minicell analysis was done. Minicells from *E. coli* P678-54 containing pMID201 or plasmids with an insertion (pMID304 [Kan^r, urease inactivated] and pMID303 [Kan^r, urease not inactivated]) were labeled with [³⁵S]methionine. Proteins were solubilized and electrophoresed on SDS-polyacrylamide gels. Autoradiographs of dried gels revealed a number of possible polypeptide subunits but the loss of only a single 25.5-kDa polypeptide by the insertionally inactivated pMID304 as compared with the urease-encoding plasmids pMID201 and pMID303 (data not shown). The site of insertional inactivation of the urease gene is shown in Fig. 1 in relation to other restriction sites and the estimated sites of λ 1105 transposon insertion (see below).

Native urease is inducible. Bacterial ureases of members of the *Proteaceae* thus far examined are inducible (29). To determine whether the native or cloned gene was inducible, *P. stuartii* BE2467 and *E. coli*(pMID201) were grown in the presence or absence of various concentrations of urea as inducer. Cell lysates from washed cells were tested for urease activity by the phenol red spectrophotometric assay (Fig. 3). Native urease was induced about fourfold when the bacteria were grown in the presence of 0.1% urea. The recombinant strain produced urease at a 10-fold-higher concentration than did uninduced *P. stuartii* BE2467 and at a 2.5-fold-higher concentration than did the induced *P. stuartii* strain. Whereas *P. stuartii* demonstrated inducibility of urease production, the recombinant strain appeared to synthesize urease constitutively.

Cellular localization of urease. To determine the cell compartment in which urease resides, cells of *P. stuartii* BE2467 and *E. coli* HB101(pMID201) were separated into cytosol, membrane, osmotic shock fluid, and supernatant fluid fractions. The urease activity of each cell fraction was determined (Table 1). In the native *P. stuartii* strain, all activity was found in the cytosol, with no trace of activity in the

TABLE 1. Cellular localization of urease

Enzyme assayed	Enzyme activity (μmol of substrate/min per mg of protein)					
	<i>P. stuartii</i> BE2467			<i>E. coli</i> HB101(pMID201)		
	Cytosol	Membrane	Periplasm	Cytosol	Membrane	Periplasm
Urease	1.9	0.0	0.0	2.8	2.5	2.5
Lactate dehydrogenase	21.4	0.0	0.0			
Beta-galactosidase				0.8	0.0	0.0
NADH dehydrogenase	20.3	217.4	0.0	9.6	67.5	0.0
Alkaline phosphatase	5.6	0.0	2.8	0.0	0.0	3.5

other fractions, including the culture supernatant. However, in the recombinant strain, significant activity was associated with all fractions.

As controls, lactate dehydrogenase and beta-galactosidase were found exclusively in the cytoplasm of *P. stuartii* (Lac⁻) and *E. coli*, respectively. NADH dehydrogenase was found predominantly associated with the membrane fractions. Alkaline phosphatase was found exclusively in the osmotic shock fluid (periplasm) of *E. coli* but was found in both the periplasm and cytosol fractions of *P. stuartii*.

Identification of urease on nondenaturing polyacrylamide gels. To identify the active urease gene products and compare the electrophoretic mobilities of the native and recombinant ureases, nondenaturing polyacrylamide gels were loaded with cell lysates from *P. stuartii* BE2467 and *E. coli* HB101(pMID101) (Fig. 4). After electrophoresis, gels equilibrated in the pH-indicating dye cresol red were exposed to a urea solution. Localized urea degradation was marked by an intense color change from yellow to red. For *P. stuartii*, a single band was observed on the activity gel (Fig. 4, lane G) that corresponded to a band produced by *E. coli*(pMID101) (Fig. 4, lane F). A more slowly migrating and more slowly developing band was observed above the primary band in this preparation and correlated with a Coomassie blue-stained protein produced by *E. coli*(pMID101) (Fig. 4, lane B) but not by plasmidless *E. coli* (Fig. 4, lane A). In

additional preparations, the more slowly migrating band was also produced by urea-induced *P. stuartii* BE2467.

Gel filtration of *P. stuartii* urease. For estimation of molecular weight, a cell lysate of *P. stuartii* was passed through a calibrated Sephacryl S-300 column. Fractions were collected, and urease activity was measured by the calibrated spectrophotometric assay (Fig. 5). A single peak was observed that corresponded to an average molecular weight of $375,000 \pm 35,000$ for four determinations. This value was in agreement with that of 380,000 determined on a Bio-Gel A (Bio-Rad Laboratories, Richmond, Calif.) 0.5 M column (data not shown). The left shoulder of the peak may correspond to the more slowly migrating band on the nondenaturing polyacrylamide gel (Fig. 4). When samples from the peak fraction (190-ml elution volume) and shoulder fraction (180 ml) were run on polyacrylamide activity gels, two urease bands were visible in lanes loaded with either of the two fractions, suggesting that the more slowly migrating activity is a form of the more rapidly migrating activity.

Spectrophotometric determination of urease activity. For rapid quantitation of urease activity, the spectrophotometric assay described by Hamilton-Miller and Gargan (13) was standardized by correlating the OD₅₆₀ of the phenol red-urea reaction mixture with pH and production of urea NH₃ as

FIG. 4. Nondenaturing polyacrylamide gel electrophoresis of recombinant and native urease. Cell lysates were loaded onto a 7% polyacrylamide (1:32, bisacrylamide:acrylamide) resolving gel with a 4% stacking gel and electrophoresed at 60 V for 16 h at 4°C. The gel was soaked in a cresol red-EDTA solution until yellow and then immersed in a 1.5% (wt/vol) urea solution. The points of red band development were marked with a punch and recorded on film, and the gel was stained with Coomassie blue (lanes A through D). Urease activity gel (lanes E through H). Lanes: A and E, *E. coli* HB101; B and F, *E. coli* HB101(pMID101); C and G, *P. stuartii* BE2467; D and H, jack bean urease. Arrow, Coomassie blue-stained band in lane B corresponding to an area of urease activity in lane F.

FIG. 5. Gel filtration of *P. stuartii* BE2467 cell lysate. A cell lysate (15 mg of protein) derived from induced cultures of *P. stuartii* BE2467 was loaded onto a Sephacryl S-300 column (2.5 by 80 cm) and eluted at a flow rate of 30 ml/h. The column was calibrated with blue dextran for void volume determination, and thyroglobulin, ferritin, catalase, aldolase, and albumin were used as molecular weight markers. The urease activity of the fractions was measured by the phenol red spectrophotometric assay.

FIG. 6. Inhibition of urease activity. Cell lysate (20 μ l; 14.4 mg of protein per ml) derived from induced cultures of *P. stuartii* BE2467 was assayed for urease activity by the phenol red-urea spectrophotometric assay. The reactions were initiated by addition of cell lysates to cuvettes containing the reaction mix and four concentrations of hydroxyurea, acetohydroxamic acid, or EDTA, all adjusted to pH 6.8. The reactions took place at 23°C in a final reaction volume of 3 ml. (A) Hydroxyurea at 0 ($\square$), 50 ($\circ$), 100 ($\diamond$), and 200 ($\triangle$) μ g/ml; (B) acetohydroxamic acid at 0 ($\square$), 50 ($\circ$), 100 ($\diamond$), and 200 ($\triangle$) μ g/ml; (C) EDTA at 0 ($\square$), 5 ($\circ$), 10 ($\diamond$), and 20 ($\triangle$) mM.

measured by the colorimetric Berthelot reaction (34). In the Berthelot reaction, the liberation of urea NH_3 by induced *P. stuartii* urease was linear with time, as measured by an increase in the OD_{560} (0.0 to 0.5 OD_{560} units). Urease activity measured by the phenol red assay showed a sigmoidal response when the OD_{560} was plotted against micromoles of NH_3 . This probably reflected the additive effect of the pH change and the buffering capacity of sodium phosphate presented in the reaction mix. The response, however, was linear between 0.15 and 0.5 OD_{560} units, allowing for direct

FIG. 7. Autoradiograph of electrophoresed [^{35}S]methionine-labeled minicell lysates from transposon insertion mutants. Minicells were isolated from *E. coli* P678-54 containing recombinant plasmids, labeled with [^{35}S]methionine, and treated at 100°C for 5 min in SDS-gel sample buffer. Solubilized proteins (4×10^5 trichloroacetic acid-precipitable cpm per lane) were electrophoresed on an 8 to 16% SDS-polyacrylamide gradient gel. The gel was fixed, stained, dried, and autoradiographed. Lanes: A, pMID402, urease positive; B, pMID403, urease negative; C, pMID401, urease negative. Upper arrow, loss of 73-kDa polypeptide in lane C; lower arrow, loss of 25-kDa polypeptide in lanes B and C. Molecular weight standards (Bio-Rad) were lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), and bovine serum albumin (66.5 kDa).

measurement of rates. No change in absorbance was detected when urea or the cell lysate from a urease-positive organism was omitted or when the cell lysate from a urease-negative organism was assayed. The phenol red spectrophotometric assay was used in the experiments described in this report because the rate of urea hydrolysis can be monitored directly over the entire course of the reaction, the assay is reproducible, and a number of assays can be monitored simultaneously.

Kinetics of urea hydrolysis. To measure the rate of urea hydrolysis and affinity for substrate, the spectrophotometric assay was used to measure the urease activity of induced cell lysates of *P. stuartii* BE2467 and *E. coli*(pMID201). Triplicate determinations of urea hydrolysis rates were performed for nine concentrations of urea ranging from 1 to 50 mM. A reciprocal plot ($1/v_0$ versus $1/[S]$) yielded a K_m of 9.4 mM urea and a V_{max} of 3.2 μ mol of NH_3 liberated per min per mg of protein for *P. stuartii*. For the recombinant strain, a K_m of 7.8 mM urea and a V_{max} of 32 μ mol of NH_3 per min per mg of protein were determined. The K_m values for the native and recombinant strains were not found to be significantly different ($P > 0.1$).

FIG. 8. Column chromatography of [³⁵S]methionine-labeled protein from minicells of *E. coli* P678-54(pMID402). Minicells from cultures (1,400 ml) of *E. coli* P678-54(pMID402) were isolated, labeled with [³⁵S]methionine, incubated in 0.5 ml of 25 mM Tris (pH 8.0)–10 mM EDTA containing lysozyme (2 mg/ml) for 30 min at 4°C, and ruptured by sonication. Insoluble material was removed by centrifugation, and labeled protein was mixed with an unlabeled urease preparation from the parental cells. Soluble protein (1.5 ml [10 mg/ml] with 10⁶ trichloroacetic acid-precipitable cpm) was loaded onto a Sephacryl S-300 column, and 8-ml fractions were collected. Urease activity and counts per minute were determined for each fraction (top panel). The fractions within each peak (A, B, or C) were pooled, dialyzed against water, and lyophilized. Protein was denatured in SDS-gel sample buffer and electrophoresed on a 10% polyacrylamide gel, which was then dried and autoradiographed (lower panel). Lanes: A, B, and C, protein from peaks A (30,000 cpm), B (22,000 cpm), and C (40,000 cpm), respectively, from the upper panel; D, denatured protein from unfractionated minicell lysate. Prestained molecular weight standards (Bethesda Research) were lysozyme (14.3 kDa), beta-lactoglobulin (18.4 kDa) alpha-chymotrypsinogen (25.7 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa) and phosphorylase B (97.5 kDa).

Inhibition of urease activity. The effects of a competitive inhibitor, a noncompetitive inhibitor, and divalent cation chelation on urease activity were measured (Fig. 6). With a substrate concentration of 35 mM urea, hydroxyurea, a competitive inhibitor, inhibited *P. stuartii* urease activity by

about 50% at 100 μg/ml (Fig. 6A). Inhibition by acetohydroxamic acid was about 50% at only 50 μg/ml and was nearly 100% at 200 μg/ml (Fig. 6B). EDTA at 20 mM inhibited activity by more than 50% (Fig. 6C).

Subunits of urease. To determine the apparent molecular weights of *P. stuartii* urease subunits, the electrophoretic mobilities of plasmid-encoded polypeptides were determined. Minicells isolated from *E. coli* P678-54 bearing λ1105-generated transposon insertions of pMID204 (Fig. 1) were radiolabeled with [³⁵S]methionine. Denatured polypeptides from minicell lysates were electrophoresed on an SDS-polyacrylamide gel and autoradiographed (Fig. 7). A transposon insertion that did not inactivate the urease gene mapped within the vector and encoded polypeptides shown in Fig. 7, lane A. An insertion that mapped 1.0 kb from the unique *Pst*I site (pMID403) was urease negative and led to the loss of production of a 25.5-kDa polypeptide (Fig. 7, lane B). The insertion which mapped 4.5 kb from the *Pst*I site (pMID401) also inactivated urease and eliminated production of 73- (Fig. 7, lane C) and 25.5-kDa polypeptides.

An alternative approach to determining subunit composition was also used. Minicells were isolated from urease-positive *E. coli* P678-54(pMID402), and plasmid-encoded proteins were labeled with [³⁵S]methionine. The minicells were treated with lysozyme-EDTA and ruptured by sonication. Soluble radiolabeled protein was spiked with an unlabeled urease preparation, and the mixture was passed through the Sephacryl S-300 column. Fractions were assayed for radioactivity and urease activity (Fig. 8). The fractions were pooled within each peak (A, B, or C), dialyzed against deionized water, and lyophilized. Protein was denatured in SDS-gel buffer and electrophoresed on SDS-polyacrylamide gels. The gels were dried and autoradiographed. The peak of radioactivity, which corresponded to fraction of peak urease activity (Fig. 8, peak A), revealed a single polypeptide band with an apparent molecular weight of 73,000. The 25.5-kDa polypeptide was not visible in any fraction. The major polypeptide with a molecular weight of 58,000 was found in peak C (Fig. 8) and was not associated with urease activity.

DISCUSSION

Urease production is a variable phenotype in *P. stuartii* and is encoded by plasmid DNA (10, 24). We described the cloning of a fragment encoding the urease gene from a large conjugative plasmid isolated from a *P. stuartii* strain and the expression of enzyme activity in *E. coli*. The enzyme appears to be unique to the *Proteaceae* tribe. With a molecular weight of 375,000, it is much smaller than those of other members of the *Proteaceae* (reported values of 560,000 to 800,000) including *P. mirabilis*, *P. vulgaris*, *M. morganii*, and *P. rettgeri* (30). The enzyme, however, is larger than that reported for "*Klebsiella aerogenes*" (*Klebsiella pneumoniae*) or *Arthrobacter oxydans* (33). The *P. stuartii* urease has a *K_m* similar to that reported for *P. vulgaris* and *P. mirabilis* but different from that of *M. morganii* and *P. rettgeri*. In addition, the enzyme pattern on nondenaturing polyacrylamide gels appears to be much simpler (two bands for *P. stuartii*) than that reported by Senior et al. (34) for other *Proteaceae* ureases. As is the case for the ureases of other species (7, 13, 29, 35, 39), the activity of the *P. stuartii* enzyme was inhibited by hydroxyurea and acetohydroxamic acid. Inhibition of activity by EDTA suggests that the enzyme requires a divalent cation (2, 6, 7, 33) for activity.

Minicell analysis of plasmid-encoded polypeptides and denaturation of partially purified urease preparations re-

vealed what may be a complex enzyme subunit structure in *P. stuartii*. Two polypeptides with apparent molecular weights of 73,000 and 25,500 were determined to be associated with the urease protein by transposon mutagenesis (Fig. 1 and 7). Insertion of a kanamycin resistance gene into a *Hind*III site inactivated urease production and also eliminated production of the 25.5-kDa polypeptide. In addition, an [³⁵S]methionine-labeled protein which comigrated with urease activity (Fig. 8) revealed a subunit size of 73 kDa upon denaturation. The production of more than one subunit is consistent with the length of DNA required to encode a functional protein. The gene sequences span at least 3.0 kb, the greatest distance between the sites of transposon insertions that inactivate urease production (pMID401 and pMID403; Fig. 1), but not more than 6.2 kb, the distance between the site of insertion of urease-positive pMID404 (Fig. 1) and the unique *Pst*I site which delineates cloned sequences from vector DNA. Localization studies demonstrated that the protein is found exclusively in the cytoplasm of native *P. stuartii*. This is consistent with the fact that multimeric proteins are not often secreted from gram-negative bacilli. The elevated production of urease in the recombinant strain may have reflected the higher number of copies of the gene sequences generated by the recombinant plasmid in *E. coli*. Constitutive production of urease by the recombinant strain may suggest a loss of regulatory sequences during subcloning.

The observation that the urease of *P. stuartii* is encoded by plasmid DNA may be of considerable importance. *P. stuartii* is a common isolate from the urine of long-term catheterized patients and, indeed, in a study by our group of bacteriuria in chronically catheterized patients, 761 (62%) of 1,230 urine specimens taken weekly from 51 patients at two chronic care facilities contained *P. stuartii* at concentrations greater than 10⁵ CFU/ml (24). With an average number of 3.8 bacterial species at that concentration per specimen, the potential for the transfer of virulence factors and antibiotic resistance exists. The 82-kb conjugative R factor from *P. stuartii* BE2467 encoding urease, sucrose utilization, and resistance to kanamycin and mercuric ion was previously shown by our group to be transferable to *E. coli* (24). This suggests that, although *P. stuartii* may not be a highly virulent pathogen per se, the transfer of potential virulence factors and antibiotic resistance to other species serves to emphasize its importance.

The cloning of urease gene sequences will provide the opportunity to (i) determine the homology of the *P. stuartii* urease with those of other bacterial species by DNA hybridization, (ii) determine whether ureases are encoded by plasmid or chromosomal DNA in other species, (iii) localize DNA fragments from other species encoding urease, and (iv) construct a *P. stuartii*, *Proteus* sp., or *M. morganii* strain specifically deleted for urease gene sequences to assess the role of urease as a single virulence factor in an animal model of urinary tract infection.

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