Urease Activity of *Proteus penneri*

HARRY L. T. MOBLEY,¹* BRADLEY D. JONES,¹ AND JOHN L. PENNER²

Division of Infectious Diseases, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201,¹ and Department of Microbiology, University of Toronto, Toronto, Ontario, Canada M5S 1A1²

Received 27 July 1987/Accepted 2 September 1987

Ten strains of *Proteus penneri* isolated from geographically diverse laboratories were tested for urease activity. Cell lysates from urea-induced cells had a mean activity of 4.9 ± 4.1 μmol of NH_3 per min per mg of protein. On nondenaturing 6% polyacrylamide activity gels, the enzymes of *P. penneri* had very similar electrophoretic mobilities within species and within the *Proteus* genus but were distinct from the ureases of *Providencia* and *Morganella* species. On lower-percentage polyacrylamide, differences in mobilities of the ureases could be detected between the *Proteus* species. From representative strains, the *P. penneri* urease was found to be inducible by growth in urea and had an apparent molecular weight of $246,000 \pm 9,000$, an isoelectric point of 5.1, and a K_m for urea of 14 mM and was inhibitable by acetohydroxamic acid, hydroxyurea, and EDTA. In an in vitro model of struvite formation, a *P. penneri* strain produced abundant crystals on a glass rod submerged in synthetic urine in the absence but not presence of acetohydroxamic acid (500 $\mu\text{g}/\text{ml}$).

Proteus penneri is a newly described (7), multiple-antibiotic-resistant (1, 2, 6) bacterial species that has been isolated from a variety of clinical sources including stool, urine, and wounds (10, 14). The presence of the organism has been frequently associated with the use of an indwelling bladder catheter (10) and has been reported as the cause of urinary calculus formation in one patient with *P. penneri* bacteriuria (9). Like other *Proteus* species, *P. penneri* produces the enzyme urease.

Urease, as described for many bacterial species, is a high-molecular-weight (5, 8), multimeric, cytoplasmic (8), nickel-containing metalloenzyme (5) that catalyzes the hydrolysis of urea to yield carbon dioxide and ammonia (3). This reaction results in a rise in pH and, in urine, is accompanied by precipitation of polyvalent cations and anions to form crystalline urinary stones, principally in the form of struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) and carbonate-apatite [$\text{Ca}_{10}(\text{PO}_4\text{CO}_3\text{OH})_6(\text{OH}_2)$] (3).

The urease of *P. penneri* is an important biochemical marker in the clinical microbiology laboratory and may represent a critical virulence factor for this organism. We report the characterization of the enzyme and compare it with the ureases of closely related species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains of *P. penneri* used in this study and their sources are as follows: HSC G.V., B. Bannatyne, Hospital for Sick Children, Toronto, Ontario, Canada; CDC 2518-74 and CDC 1803-73, F. Hickman-Brenner, Centers for Disease Control, Atlanta, Ga.; PMH0748, Princess Margaret Hospital, Toronto, Ontario, Canada; TGH2583 and TGH4596, Toronto General Hospital, Toronto, Ontario, Canada; A9/01 and PH7713, P. M. Hawkey, Bristol, United Kingdom; ZUR1985, A. Von Gravenitz, Zurich, Switzerland; and STJ375/86, St. Joseph's Hospital, Toronto, Ontario, Canada. Strains of *Proteus mirabilis*, *Providencia stuartii*, *Providencia rettgeri*, and *Morganella morganii* were isolated from patients with catheter-associated bacteriuria (8). Strains were maintained on Luria agar by weekly passage and stored at -70°C in

Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 15% (vol/vol) glycerol.

Urease preparations. Bacteria were grown at 37°C with aeration in 100 ml of Luria broth (12) supplemented or not with 0.05% (wt/vol) urea after autoclaving. Cells were harvested by centrifugation ($7,000 \times g$, 10 min, 4°C), washed once with 20 mM sodium phosphate (pH 6.8), suspended in 5 ml of 20 mM sodium phosphate (pH 6.8), and ruptured in a precooled French pressure cell (SLM Instruments, Inc./American Instrument Co., Urbana, Ill.) at $20,000 \text{ lb}/\text{in}^2$. The lysate was centrifuged at $27,000 \times g$ for 30 min, and the supernatant was removed with a Pasteur pipette and used directly or stored in 1-ml portions at -70°C . Protein was determined by the method of Lowry et al. (11) by using bovine serum albumin as a standard.

Spectrophotometric urease assay. Rates of urea hydrolysis were measured by the spectrophotometric assay of Hamilton-Miller and Gargan (4) and as described previously (8, 13). The assay was calibrated by comparing changes in optical density at 560 nm with ammonia liberation as measured with an ammonia electrode (Corning Glass Works, Corning, N.Y.) connected to a pH meter (model $\phi 71$, Beckman Instruments, Inc., Fullerton, Calif.) which was calibrated with 10^{-6} to 10^{-1} M ammonium chloride according to the instructions of the manufacturer. The effect of urease inhibitors was assessed by the addition of enzyme preparations to urease assay buffer containing various concentrations of inhibitor. Stock solutions of inhibitors were adjusted to pH 6.8 before being added.

Column chromatography. Urease preparation (1 ml of 10 to 15 mg of protein per ml) was loaded onto a Sephacryl S-300 column (100 by 2.5 cm) equilibrated with 20 mM sodium phosphate-0.02% sodium azide (pH 6.8). Fractions (3 ml) were collected at a flow rate of 30 ml/h. The column was calibrated with standard proteins of known molecular size (in daltons): thyroglobulin, 669,000; ferritin, 440,000; catalase, 232,000; and aldolase, 158,000. Void volume was determined with Blue Dextran (Pharmacia Biotechnology Products, Piscataway, N.J.). Fractions with peak activity were identified by adding 100 μl of each fraction to wells of microtiter dishes containing 100 μl of 0.04% cresol red-100 mM urea. Activity of peak fractions, indicated by a change in color of solution from yellow to red within 30 min, was

* Corresponding author.

then quantitated by the spectrophotometric assay. Determinations were made in triplicate.

Nondenaturing polyacrylamide gel electrophoresis. Urease preparations (20 μ l) were mixed with 20 μ l of 50% sucrose–0.1% bromphenol blue, loaded onto a 5.5 or 6% polyacrylamide gel (16 by 16 by 0.15 cm) (1:32 [vol/vol] bisacrylamide to acrylamide; United States Biochemical Corp., Cleveland, Ohio) with a 4% stacking gel, and electrophoresed for 4 h at 250 V. Gels were equilibrated with 0.02% cresol red–0.1% EDTA (16) and then immersed in 1.5% (wt/vol) urea. Urease activity, indicated by rapid color development, was recorded on Plus-X Pan film (Eastman Kodak Co., Rochester, N.Y.).

Isoelectric focusing. Gels were prepared as described for the LKB Ultramould system (LKB Instruments, Inc., Gaithersburg, Md.). Urease preparations (30 μ l) were loaded approximately 2 cm from the cathode onto a 5% polyacrylamide gel (100 by 245 by 0.1 mm) (1:32 [vol/vol] bisacrylamide to acrylamide; United States Biochemical) with 3.0% ampholytes (pH range, 3.5 to 9.5; LKB Instruments). Gels were electrophoresed at a constant power setting of 3 W for 6 h and cooled with circulating ice water. Ferritin (100 to 150 μ g) was used as a focusing standard. The pH gradient was measured with a surface electrode (Beckman), and the gel was equilibrated in 0.02% (wt/vol) cresol red–0.1% (wt/vol) EDTA. Points of migration of ureases (isoelectric points) were determined by immersion of the gel in 1.5% urea as described above for nondenaturing polyacrylamide gels.

Crystallization in synthetic urine. Sterile Erlenmeyer flasks (125 ml) were fitted with rubber stoppers through which glass rods (0.5-cm outside diameter) were inserted. The flasks were filled with 100 ml of filter-sterilized synthetic urine (3) supplemented with 10% (vol/vol) Luria broth (12). Acetohydroxamic acid (AHA) was added to one flask at a concentration of 500 μ g/ml. Flasks were inoculated with 3×10^8 CFU of *P. penneri* PH7713 suspended in 1 ml of synthetic urine and were incubated statically for 48 h at 37°C. Results were recorded on Plus-X Pan film (Kodak), and pH of the solution was measured with a combination pH electrode.

RESULTS

Urease preparations from the 10 *P. penneri* isolates were electrophoresed on a nondenaturing polyacrylamide gel and assayed for electrophoretic mobility of the enzyme (Fig. 1A). All 10 strains produced an enzyme with a rapidly developing lower band followed by a more slowly developing upper band. Ureases from all strains had electrophoretic mobilities that were not significantly different. When compared with ureases of other *Proteeae* genera (Fig. 1B) on a 6% gel, *P. penneri* urease had an electrophoretic pattern similar to that of *Proteus vulgaris* GO1232 and *P. mirabilis* BU7354 but distinct from that of *P. mirabilis* BR2528 (found to produce two distinct enzymes [8]), *Providencia stuartii* BE2467, and *Providencia rettgeri* TA1738. When profiles were compared on a 5.5% gel, the ureases of *P. penneri*, *P. vulgaris*, and *P. mirabilis* demonstrated similar but distinct electrophoretic mobilities (data not shown). In addition, the urease of *M. morganii*, a very-high-molecular-weight enzyme which has a low mobility, was very distinct from all *Proteus* enzymes. The *P. penneri* urease was found by isoelectric focusing to have an isoelectric point of 5.1.

To estimate a molecular size for the intact enzyme, urease preparations from urea-induced cell lysates of strain PH7713 were fractionated on a Sephacryl S-300 gel filtration column

(Fig. 2). When compared with the elution profiles of standard proteins, fractions with peak urease activity corresponded to an apparent molecular size of $246,000 \pm 9,000$ daltons.

The urease of *P. penneri* was inducible. When strain PH7713 was grown in Luria broth with no urea, urease activity was found to be 0.7 μ mol of NH_3 per min per mg of protein for triplicate determinations. When grown with 0.05% urea, activity was 4.8 μ mol of NH_3 per min per mg of protein, a sevenfold increase. Similarly, strain TGH4596, when uninduced, hydrolyzed urea at a rate of 2.4 μ mol of NH_3 per min per mg of protein, but when induced, increased the rate to 5.3, a twofold increase.

Activities of urease preparations from induced cells were quantitated by the spectrophotometric assay (Fig. 3B). For the 10 strains of *P. penneri*, the mean activity was 4.9 ± 4.1 μ mol of NH_3 per min per mg of protein at 50 mM urea at 23°C. Kinetics of urea hydrolysis were measured for strain PH7713 (Fig. 3A). Rates of hydrolysis were measured at urea concentrations of 1, 2, 5, 10, 20, and 50 mM. A double-reciprocal plot of 1/ μ mol of NH_3 per min per mg of protein versus 1/mM urea (inset) revealed a K_m for urea of 14 mM.

The urease of *P. penneri* can be specifically inhibited by a variety of compounds. Enzyme activity of urease preparations from strain TGH4596, induced with urea, was measured in the presence and absence of inhibitors at a saturating urea concentration of 28 mM. Concentrations that inhibited activity by 50% were calculated. AHA, a noncom-

FIG. 1. Urease activity in nondenaturing polyacrylamide gels of cell lysates from *Proteus* and *Providencia* species. Soluble protein derived from induced cell lysates was loaded onto a 5.5% polyacrylamide gel and was electrophoresed. The gel was equilibrated with cresol red and then was transferred to a 1.5% urea solution. Bands are the result of an intense color development at the points of migration of ureases. (A) *P. penneri* strains. Lanes: 1, HSC G.V.; 2, CDC 2518-74; 3, CDC 1808-73; 4, STJ375/86; 5, A9/01; 6, PH7713; 7, ZUR1985; 8, TGH2583; 9, TGH4596. A tenth strain, PMH0748, was too faint to record. (B) Lanes: 1, *P. penneri* PH7713; 2, *P. vulgaris* GO1232; 3, *P. mirabilis* BR2528; 4, *P. mirabilis* BU7354; 5, *Providencia stuartii* BE2467; 6, *Providencia rettgeri* TA1738.

FIG. 2. Gel filtration of *P. penneri* cell lysate. Urease preparations from urea-induced cells of *P. penneri* PH7713 were fractionated on a Sephacryl S-300 column. Void volume was determined by elution of Blue Dextran. The column was calibrated with standard proteins which eluted at volumes indicated by the arrows. Urease activities of the fractions were measured by the spectrophotometric assay.

petitive inhibitor, was found to inhibit at a concentration of 8.5 μg/ml (113 μM). The urea analog hydroxyurea, a competitive inhibitor, required 32 μg/ml (420 μM) for 50% inhibition. EDTA, a divalent cation chelator, reduced activity by one-half at 2.8 mg/ml (7.5 mM).

To demonstrate that the urease of *P. penneri* was capable

of inducing crystal formation, flasks containing synthetic urine were inoculated with *P. penneri* TGH4596, allowed to incubate statically, and observed after 48 h (Fig. 4). Abundant crystals formed on the glass rod and on the bottom of the flask that contained no AHA (Fig. 4A). However, in the presence of AHA (500 μg/ml), although growth of the

FIG. 3. Kinetics of urea hydrolysis by *P. penneri* urease. (A) Rates of urea hydrolysis were measured for six concentrations of substrate. Cell lysate (20 μl of 15 mg of protein per ml) derived from induced cultures of *P. penneri* PH7713 was assayed by the phenol red spectrophotometric assay in a 3-ml reaction volume at 23°C. Rates were derived from linear portions of the curves. A reciprocal plot of these data is inset. Data represent means of three assays. (B) Datum points represent the urease activities of each of the 10 strains listed in Table 1. Activity was measured spectrophotometrically at a urea concentration of 50 mM at 23°C and represents triplicate measurements.

FIG. 4. Crystal formation in synthetic urine inoculated with *P. penneri*. A flask containing synthetic urine (3) supplemented with 10% Luria broth was inoculated with *P. penneri* PH7713 and incubated statically at 37°C for 48 h. A glass rod served as a nidus for crystal formation. (A) Control culture; (B) supplemented with urease inhibitor AHA (500 µg/ml).

organism was not affected, no visible crystals formed (Fig. 4B). The final pH of the suspension containing no AHA was 9.2, whereas the flask with AHA had a pH of 6.1.

DISCUSSION

The ureases of *P. penneri* appear to represent a homogeneous group of enzymes within the species and are similar to the enzymes of some strains of *P. mirabilis* and *P. vulgaris*. The enzyme is a high-molecular-weight protein that requires a divalent cation for activity, as indicated by inhibition with EDTA. On the basis of other biochemical parameters such as electrophoretic mobilities on gels with a variety of polyacrylamide concentrations, molecular weights by gel filtration, isoelectric points, kinetics of urea hydrolysis, and patterns of inhibition by specific compounds, the urease of *P. penneri* is unique when it is compared with the enzymes of *P. mirabilis*, *P. vulgaris*, *Providencia stuartii*, *Providencia rettgeri*, *M. morganii* (8), and a non-*Proteaeae* species, *Campylobacter pylori* (H. L. T. Mobley and B. D. Jones, unpublished observation).

Although there are only a few reports of the pathogenic role of this newly described species, it has been identified frequently as a urinary tract isolate (10) with hemolytic activity and the ability to invade Vero cells in culture (15). Of particular interest is the report of a patient with persistent *P. penneri* bacteriuria that was accompanied by urinary struvite-uric acid stone formation. The organism, in fact, was isolated from the center of the stone and perhaps served as the nidus for crystallization. Since urease is the primary cause of infection-induced stones (3), it is likely that the relatively active urease of *P. penneri* elevated the pH of the urine and induced precipitation of polyvalent ions to form the stone. Although we found that the urease of *P. penneri*

does not have particularly high affinity for substrate (K_m of 14 mM), the enzyme in urine is saturated at least 10-fold (average urea concentration in urine is 400 mM) and thus is working at maximum velocity at levels comparable to those of *P. vulgaris* but less than those of *P. mirabilis* (8). Also, urea concentrations in commercial urea broth and agar base are greater than 300 mM, thus providing saturating conditions for maximum urease activity. Under these conditions, a strong urease reaction should generally be observed with most isolates of *P. penneri*.

It is likely that as the taxonomy of this organism is refined, *P. penneri* will be more frequently reported by clinical microbiology laboratories. Furthermore, the association of this organism with urinary tract, bladder, and kidney stones, a result of urease activity, will undoubtedly increase.

ACKNOWLEDGMENTS

We thank Linda Horne for expert manuscript preparation.

This work was supported by Public Health Service grants R29 AI23328 and PO1 AGO4393 from the National Institutes of Health.

LITERATURE CITED

1. Fuksa, M., S. Krajden, and A. Lee. 1984. Susceptibilities of 45 clinical isolates of *Proteus penneri*. *Antimicrob. Agents Chemother.* 26:419-420.
2. Grace, M. E., F. J. Gregory, P. P. Hung, and K. P. Fu. 1986. Purification and properties of a β -lactamase from *Proteus penneri*. *J. Antibiot.* 39:938-942.
3. Griffith, D. P., D. M. Musher, and C. Itin. 1976. Urease: the primary cause of infection-induced urinary stones. *Invest. Urol.* 13:346-350.
4. Hamilton-Miller, J. M. T., and R. A. Gargan. 1979. Rapid screening for urease inhibitors. *Invest. Urol.* 16:327-328.
5. Hausinger, R. P. 1987. Nickel utilization by microorganisms. *Microbiol. Rev.* 51:22-42.
6. Hawkey, P. M., S. J. Pedler, and A. Turner. 1983. Comparative in vitro activity of semisynthetic penicillins against *Proteaeae*. *Antimicrob. Agents Chemother.* 23:619-621.
7. Hickman, F. W., A. G. Steigerwalt, J. J. Farmer III, and D. J. Brenner. 1982. Identification of *Proteus penneri* sp. nov., formerly known as *Proteus vulgaris* indole negative or as *Proteus vulgaris* biogroup 1. *J. Clin. Microbiol.* 15:1097-1102.
8. Jones, B. D., and H. L. T. Mobley. 1987. Genetic and biochemical diversity of ureases of *Proteus*, *Providencia*, and *Morganella* species isolated from urinary tract infection. *Infect. Immun.* 55:2198-2203.
9. Krajden, S., M. Fuksa, W. Lizewski, L. Barton, and A. Lee. 1984. *Proteus penneri* and urinary calculi formation. *J. Clin. Microbiol.* 19:541-542.
10. Krajden, S., M. Fuksa, C. Petrea, L. J. Crisp, and J. L. Penner. 1987. Expanded clinical spectrum of infections caused by *Proteus penneri*. *J. Clin. Microbiol.* 25:578-579.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
12. Miller, J. H. 1972. Experiments in molecular genetics, p. 433. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. Mobley, H. L. T., B. D. Jones, and A. E. Jerse. 1986. Cloning of urease gene sequences from *Providencia stuartii*. *Infect. Immun.* 54:161-169.
14. Muller, H. E. 1986. Occurrence and pathogenic role of *Morganella-Proteus-Providencia* group bacteria in human feces. *J. Clin. Microbiol.* 23:404-405.
15. Rozalski, A., and K. Kotelko. 1987. Hemolytic activity and invasiveness in strains of *Proteus penneri*. *J. Clin. Microbiol.* 25:1094-1096.
16. Senior, B. W., N. C. Bradford, and D. S. Simpson. 1980. The ureases of *Proteus* strains in relation to virulence for the urinary tract. *J. Med. Microbiol.* 13:507-512.