Purification, Characterization, and Genetic Organization of Recombinant *Providencia stuartii* Urease Expressed by *Escherichia coli*†

SCOTT B. MULROONEY,¹ MARTHA J. LYNCH,² HARRY L. T. MOBLEY,² AND ROBERT P. HAUSINGER^{1,3*}

¹Department of Biochemistry and ²Department of Microbiology and Public Health, ³Michigan State University, East Lansing, Michigan 48824, and Division of Infectious Diseases, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201²

Received 21 December 1987/Accepted 22 February 1988

Recombinant urease from *Providencia stuartii* has been expressed in and purified from *Escherichia coli*, and the genetic organization of the structural genes has been determined. Urease expression was induced by urea and repressed by nitrogen-rich components in the medium. The urease protein was purified 331-fold by DEAE-Sephadex, phenyl-Sephadex, Mono-Q, and phenyl-Superose chromatographies with a 7.3% yield. The enzyme possessed a K_m for urea of 9.3 mM and hydrolyzed urea at a V_{max} of 7,100 $\mu\text{mol/min per mg}$. *P. stuartii* urease is composed of three polypeptides (M_r s, 73,000, 10,000, and 9,000) denoted by α , β , and γ . The native enzyme is best described as $(\alpha_1\beta_2\gamma_2)_2$, based on a native M_r of 230,000, obtained by gel filtration chromatography, and on the Coomassie blue staining intensities of the individual subunits. Atomic absorption analysis of the pure protein revealed 1.9 ± 0.1 nickel ions per $\alpha_1\beta_2\gamma_2$ unit. In vitro transcription-translation analysis of transposon insertion mutants of the recombinant urease demonstrated that the urease peptides are encoded on adjacent DNA sequences and transcribed as a polycistronic mRNA in the order γ , β , and then α . Three urease-defective insertion mutants were identified that did not affect synthesis of urease subunit polypeptides, indicating that some nickel processing, enzyme activation, or other function may also be necessary for producing an active urease.

Urease, a nickel-containing enzyme that catalyzes the hydrolysis of urea to carbon dioxide and ammonia, is synthesized by a wide variety of bacteria, fungi, and plants (1, 3, 11, 12). Bacterial urease may be a contributing factor in the development of pyelonephritis (5, 16, 22), hyperammonemia (23), and catheter encrustation (20), as well as kidney and bladder stone formation (9, 21). In the last case, ammonia generated from urea hydrolysis alkalinizes urine, resulting in the precipitation of polyvalent anions and cations in the form of struvite and apatite salts (9). Although stones may develop from other causes, it has been estimated that 20 to 40% of all urinary stones are due to urease-positive bacteriuria (21).

In chronically catheterized patients, there is a high incidence of bacteriuria, with the organism *Providencia stuartii* a prevalent isolate (26, 27). The urease genes of this microbe are located on a large conjugative plasmid in a number of isolates (8, 18) and have been cloned and expressed in *Escherichia coli* (19). Preliminary minicell analysis of insertion and transposon mutants of the recombinant urease revealed that a region comprising 3.0 to 6.2 kilobase pairs (kb) of DNA was necessary for urease activity and encoded at least two polypeptides (M_r s, 73,000 and 25,500) (19). The kinetic parameters of the recombinant enzyme were similar to those of the native strain (19).

In addition to the urease genes of *P. stuartii*, those of *Bacillus pasteurii* (13), *Proteus mirabilis* (B. D. Jones and H. L. T. Mobley, submitted for publication), *Klebsiella aerogenes* (S. B. Mulrooney and R. P. Hausinger, manuscript in preparation), *Klebsiella pneumoniae*, and *Morga-*

nella morganii (W. Nichols, G. F. Gerlach, and S. Clegg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, D115, p. 91; for the latter species, Nicholson, B. D. Jones, and H. L. T. Mobley, manuscript in preparation) have been cloned in *E. coli* by selecting for a urease-positive phenotype. However, no detailed studies of urease expression, enzyme characterization, protein purification, or gene-polypeptide assignments have been reported for any recombinant urease.

It was recently shown that the purified ureases of several bacterial species are composed of one large and two small polypeptides (25), which contrasts with the homomeric structure of the jack bean enzyme (1, 3). This multimeric structure may be a general property of bacterial ureases. Examination of the urease gene sequences should provide information about the arrangement of the DNA sequences encoding the multiple subunits, the nature and function of each subunit, and the regulation of urease expression.

This report extends the work of Mobley et al. (19) in which *P. stuartii* urease genes were cloned and some aspects of urease expression in *E. coli* were investigated. A purification scheme for recombinant urease is provided, the properties of recombinant urease are described, and the genetic localization and organization of the structural genes are presented.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* HB101 (*recA pro leu rpsL hsdS*) containing plasmid pMID201 (*ure⁺ tet*) or pMID204 (*ure⁺*), which possess urease genes cloned from a conjugative plasmid of *P. stuartii* BE2467 (19), were used for these experiments. The medium used (17) was either LB broth or ammonia-free M9 minimal medium supplemented with 5 or 10% (vol/vol) LB broth. When required, 1 ml of trace mineral solution (24) and 15 ml of filter-sterilized 1 M urea per liter were added. For growth of *E.*

* Corresponding author.

† Article no. 12549 from the Michigan State University Agricultural Experiment Station.

coli HB101(pMID201), all cultures contained 10 µg of tetracycline per ml.

Regulation of urease expression. For urease expression studies, 125-ml Erlenmeyer flasks containing 20 ml of medium were inoculated with 5 µl of a stationary LB culture and were grown overnight in a 37°C water bath with rapid shaking. Aliquots (5 ml) of the cultures were chilled on ice, centrifuged, washed twice with an equal volume of ammonia-free M9 salts (M9 without glucose and ammonium chloride), and suspended in a final volume of 2.5 ml. The cells were sonicated four times for 30 s each time with a sonicator (Sonic Dismembrator; Fisher Scientific Co., Livonia, Mich.) by using a small probe (4-mm-diameter tip) at 30% power. A 1-ml portion of the extract was centrifuged for 5 min at 4°C in a microcentrifuge (Eppendorf; Brinkmann Instruments, Inc., Westbury, N.Y.), and the supernatant was assayed for urease activity.

Assays. Urease activity was determined by monitoring the rate of ammonia released from urea by formation of indophenol, which was measured at 625 nm (28). The assay buffer consisted of 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.), 50 mM urea, and 0.5 mM EDTA (pH 7.75). One unit of urease activity is defined as the amount of enzyme required to hydrolyze 1 µmol of urea per min at 37°C under the assay conditions described above. Protein was measured by the method of Lowry et al. (15) by using bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis. All electrophoresis was carried out by using the buffers of Laemmli (14), except that sodium dodecyl sulfate was omitted for native gels. Denaturing gels were run by using either 16% polyacrylamide (bisacrylamide-acrylamide, 1:32) resolving gel with a 5% stacking gel or a 10 to 15% polyacrylamide gradient resolving gel with a 4.5% stacking gel. Samples were run after denaturing at 100°C for 5 min. The gels were stained with Coomassie brilliant blue (Sigma) and scanned by using a Gilford Response spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 562 nm. Nondenaturing gels were run by loading 1 U of urease activity on a 3% stacking gel and a 6% resolving gel, which was then stained for activity by using a phenol red indicator, similar to the method of Blattler et al. (4).

Large-scale growth and urease purification. *E. coli* HB101 (pMID201) was grown in ammonia-free M9 minimal medium supplemented with 10% LB broth, 15 mM urea, 10 µg of tetracycline per ml, and 1 ml of trace mineral solution per liter. Cultures were grown at 37°C in either a 25-liter Microferm fermentor (New Brunswick Scientific Co., Edison, N.J.) (15-liter cultures) with rapid mixing and aeration or in a 20-liter bottle (containing a 10-liter culture) immersed in a 37°C bath without shaking but with vigorous sparging. Cells were harvested by using a Pellicon concentrator (Millipore Corp., Bedford, Mass.), washed once with PEB buffer (20 mM phosphate, 1 mM EDTA, 1 mM 2-mercaptoethanol [pH 7.0]), resuspended in an equal volume of PEB buffer, and frozen at -20°C. The cells were thawed, disrupted by two passes through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 16,000 lb/in², and centrifuged at 100,000 × *g* for 60 min at 4°C. DEAE-Sepharose and phenyl-Sepharose chromatographies were performed on conventional columns at 4°C. All subsequent purification steps were carried out on a Fast Protein Liquid Chromatography system (Pharmacia, Uppsala, Sweden) at room temperature. All resins and columns were purchased from

Pharmacia. PEB buffer with the stated additions was used in all phases of the purification.

Urease characterization. The reaction rates for purified urease were measured as the concentration of urea was varied from 1 to 100 mM, and the data were analyzed by the method of Wilkinson (29).

The molecular weight for native *P. stuartii* urease was estimated by using Superose 6 gel filtration chromatography in PEB buffer containing 0.1 M KCl. The column (1.0 by 30 cm) was standardized by using thyroglobulin, gamma globulin, ovalbumin, myoglobin, and vitamin B-12 (*M_r*s, 670,000, 158,000, 44,000, 17,000, and 1,350; Bio-Rad Laboratories, Richmond, Calif.).

The nickel content of the purified urease was determined by using a PE 5000 atomic absorption spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) equipped with an HGA 500 graphite furnace and an AS-1 autosampler. Samples were hydrolyzed in 1 M HNO₃, evaporated, and dissolved in 50 mM HNO₃. Nickel standards, prepared with and without bovine serum albumin (to mimic the enzyme matrix), were treated identically to the urease samples. Aliquots (20 µl) were dried at 120°C, charred at 1,200°C, atomized at 2,700°C, and quantitated for nickel by integrating the peak area while using the background correction mode.

Transposon mutagenesis. *E. coli* HB101(pMID204) was infected with lambda 467, which carries transposon Tn5, by the method of de Bruijn and Lupski (6). Kanamycin-resistant transductants were pooled and cultured in 100 ml of Luria broth containing kanamycin (50 µg/ml). Plasmid DNA was isolated by the method of Birnboim and Doly (2), purified by centrifugation to equilibrium in a CsCl gradient (17), and used to transform *E. coli* HB101. Kanamycin-resistant transformants were screened for urease activity by replica plating onto urea segregation agar (7) and by inoculating microtiter plates containing 0.04% cresol red-100 mM urea. The site of transposon insertion in pMID204 was estimated from single digests of plasmid DNA with *Hind*III and *Ava*I.

In vitro transcription-translation. In vitro transcription-translation was performed as directed by the manufacturer (Digene, College Park, Md.) with the following modifications. Each reaction tube contained 13 µCi of L-[³⁵S]methionine (1,123 Ci/mmol; New England Nuclear Corp., Boston, Mass.). After being labeled, the samples were treated at 100°C for 5 min in sodium dodecyl sulfate sample buffer (14). Solubilized proteins (1.5 × 10⁵ trichloroacetic acid-precipitable cpm per lane) were electrophoresed as described above. After electrophoresis, gels were fixed, soaked in 1% glycerol-10% glacial acetic acid, dried onto filter paper under vacuum, and autoradiographed by using XAR-2 X-ray film (Eastman Kodak Co., Rochester, N.Y.) for 22 h.

RESULTS

Optimization of urease expression. The specific activities of crude cell extracts grown under various conditions are shown in Table 1. Urease expression in *E. coli* was greatest when urea was present, consistent with regulation by urea induction. In addition, urease expression was repressed in very rich medium (LB broth), but the enzyme was synthesized when the amount of nitrogen-rich constituents in the medium decreased to 5 or 10% the amount in LB broth. The presence or absence of trace minerals had little effect. When a growth curve was performed by using the medium described for large-scale growth, the specific activity of the culture was highest during the early exponential phase, after which it gradually subsided (data not shown).

TABLE 1. Optimization of recombinant *P. stuartii* urease expression by *E. coli*(pMID201)^a

Medium ^b	Presence of:		A_{600} ^d	Sp act ^e
	15 mM urea	Trace minerals ^c		
LB	—	—	0.79	0.0
LB	+	—	0.69	0.8
LB	+	+	0.66	3.4
M9 + 10% LB	—	—	0.53	0.0
M9 + 10% LB	—	+	0.51	0.0
M9 + 10% LB	+	+	0.51	68.0
M9 + 10% LB	+	3+ ^f	0.80	75.0
M9 + 5% LB	—	—	0.54	2.5
M9 + 5% LB	—	+	0.53	2.7
M9 + 5% LB	+	+	0.40	54.0

^a *E. coli* HB101 is nonureolytic.^b See reference 17. All cultures contained tetracycline (10 µg/ml).^c See reference 24.^d A_{600} for the culture at time of harvest.^e Specific urease activity of extracts from disrupted cells expressed in µmol of urea per min per mg.^f Three times the normal amount of trace minerals was added.

Purification of urease. The crude extract from 31 g (wet weight) of cells was applied to a DEAE-Sepharose column (2.5 by 15 cm) equilibrated with PEB buffer. The urease was eluted with a 400-ml linear gradient of 0 to 1.0 M KCl in PEB buffer, resulting in a single peak of activity at 350 mM KCl. Peak fractions were adjusted to 1.0 M KCl and loaded onto a preequilibrated phenyl-Sepharose column (1.5 by 14 cm). After a wash with 80 ml of 1.0 M KCl in PEB buffer, the urease was removed with a single-step elution using 80 ml of PEB buffer. Washing of the column with 20% dimethyl sulfoxide in PEB buffer eluted only trace amounts of additional urease activity. Peak phenyl-Sepharose fractions were combined, diluted with an equal volume of PEB buffer, and applied to a Mono-Q HR 5/5 Fast Protein Liquid Chromatography column. The activity was eluted as a doublet at 350 mM KCl by using a linear KCl gradient in PEB buffer. The active fractions were pooled, adjusted to 2.0 M KCl, and loaded onto a phenyl-Superose HR 5/5 Fast Protein Liquid

TABLE 2. Purification of recombinant *P. stuartii* urease from *E. coli*(pMID201)

Purification step	Sp act (µmol of urea/min per mg)	Purification (fold)	Total activity (µmol/min)	Total protein (mg)	Recovery (%)
Crude extract	16.7	1	69,000	4,180	100
DEAE-Sepharose	190	11	45,300	239	67.5
Phenyl-Sepharose	881	53	33,500	38.0	48.6
Mono-Q	2,290	137	19,600	8.6	28.4
Phenyl-Superose	5,520	331	4,990	0.9	7.2

Chromatography column. The protein was eluted with a descending 2.0 to 0 M KCl gradient in PEB buffer, yielding a major activity peak at 1.1 M KCl and a minor activity peak at 0.3 M KCl (Fig. 1). The latter peak was composed of both urease and contaminating protein, presumably as an aggregate. Fractions in the major peak were pooled and concentrated by ultrafiltration (Centricon 10; Amicon Corp., Lexington, Mass.). The purification procedure and results are summarized in Table 2.

Analysis of urease by gel electrophoresis. Denatured samples of purified urease were electrophoresed by using a sodium dodecyl sulfate–10 to 15% polyacrylamide gradient gel (Fig. 2). Three polypeptides were observed in the pure recombinant protein; they were similar to those found in several other bacterial ureases (25). Subunit ratios derived from scanning densitometry of the Coomassie blue-stained bands after normalization for molecular weights were 1:1.7:1.9 for the bands with M_s of 73,000, 10,000, and 9,000, respectively. These peptides, in decreasing order of size, are designated α , β , and γ .

Samples of *P. stuartii* urease were run on nondenaturing gels and stained for activity. The final pure protein gave a single sharp band, whereas samples of the preparation taken at earlier stages in the purification showed several additional faint, more-slowly-migrating bands.

Kinetic parameters. A K_m of 9.3 ± 1.2 mM and a V_{max} of $7,100 \pm 300$ µmol of urea per min per mg were obtained for purified *P. stuartii* urease. This K_m value compares quite

FIG. 1. Phenyl-Superose Fast Protein Liquid chromatography of recombinant *P. stuartii* urease expressed by *E. coli*(pMID201). Active fractions from Mono-Q chromatography were pooled, adjusted to 2 M KCl, and chromatographed as described in the text by using a KCl gradient (---). Aliquots of the 1.0-ml fractions were assayed for urease activity (■), and A_{280} was monitored (—).

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gradient gel of purified recombinant *P. stuartii* urease expressed in *E. coli*. A sample of the purified enzyme was run as described in the text by using 8 μ g of protein, and the gel was stained with Coomassie brilliant blue. The standards (stds) used were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme (Bio-Rad Laboratories, Richmond, Calif.), myoglobin, and myoglobin cyanogen bromide fragments I-II, I, and II (Sigma). Numbers on the right indicate molecular weights.

well with that reported for crude cell extracts from *P. stuartii* or from *E. coli* expressing recombinant urease (19).

Native molecular weight. The native molecular weight for the purified *P. stuartii* urease was estimated as $230,000 \pm 20,000$ by Superose 6 gel filtration chromatography. This value is significantly less than the value of 337,000 reported for crude cell extracts (19). This discrepancy may be due to association with other proteins in the more crude preparation.

Nickel analysis. Atomic absorption analysis showed that urease had 1.9 ± 0.1 mol of nickel ion per mol of $\alpha_1\beta_2\gamma_2$ structural unit when compared with a standard curve prepared as described in Materials and Methods.

Transposon mutagenesis. Polypeptides encoded by plasmid pMID204 mutagenized by transposon insertions at various sites were labeled with [35 S]methionine by in vitro transcription-translation. Autoradiographs of electrophoresed polypeptides (Fig. 3) revealed that transposon insertions that did not inactivate urease result in the synthesis of polypeptides with apparent molecular weights of 73,000, 10,000, and 9,000, corresponding to subunits observed in the purified enzyme (Fig. 2).

Seventeen unique sites of transposon insertions into the urease-encoding plasmid pMID204 were mapped (Fig. 4). Insertions resulting in inactivation of urease activity (from pMID540 to pMID504) spanned a 4.4-kb region. The first site of insertion that did not inactivate urease synthesis (pMID 506) was estimated to be 6.0 kb from the insert-vector junction marked by the unique *Pst*I site. Synthesis of urease structural subunits was interrupted (noted by the lack of synthesis or truncation of the polypeptide) by insertions that mapped 2.4 kb apart (pMID540 to pMID548). In six cases (pMID554, pMID586, pMID516, pMID503, pMID517, and

pMID548), the insertions resulted in the loss of only the 73-kilodalton (kDa) polypeptide, whereas for pMID530, the 73- and 10-kDa polypeptides were lost and for pMID540 and pMID551, all three polypeptides were lost. One insertion (pMID504) resulted in the inactivation of urease but did not interrupt synthesis of structural polypeptides of the enzyme.

DISCUSSION

Growth of *E. coli* containing the *P. stuartii* urease genes shows that urease expression is induced by urea, as reported for wild-type *P. stuartii* (19). This result differs from that of Mobley et al. (19), who reported that the expression of cloned urease gene sequences appeared to be constitutive for recombinant cells grown in L broth. Nitrogen-rich conditions (e.g., LB medium) repressed urease expression in the experiments described above. Urease regulation by induction and repression indicates that the cloned segment must still possess regions necessary for regulating urease expression. In addition, *E. coli* regulatory components are probably necessary. The fact that omission of the trace mineral solution still resulted in high specific activities shows that there is enough trace nickel in the medium constituents to support active urease production. An ammonia-free M9 medium supplemented with 10% LB broth, trace minerals, and 15 mM urea was selected for large-scale culturing; it was low enough in available nitrogen sources to promote high levels of urease, yet it allowed the cells to grow rapidly for fast culturing of cells.

FIG. 3. Autoradiograph of electrophoresed 35 S-labeled proteins from in vitro transcription-translation of transposon insertion mutants. Plasmid DNA was purified from isolates of *E. coli* HB 101(pMID204::Tn5). Proteins translated in the presence of [35 S]methionine by using an in vitro transcription-translation preparation were solubilized and electrophoresed on a 14% polyacrylamide gel. The gel was fixed, dried, and autoradiographed. Lanes: A, pMID506, urease positive; B, pMID540, urease negative; C, pMID587, urease negative; D, pMID530, urease negative; E, pMID 554, urease negative; F, pMID516, urease negative; G, pMID548, urease negative; H, pMID504, urease negative; I, pMID501, urease positive; J, pMID204, parent plasmid, urease positive. Upper arrow, Loss of the 73-kDa polypeptide in lanes B through G; middle arrow, loss of the 10-kDa polypeptide in lanes B through D; lower arrow, loss of the 9-kDa polypeptide in lanes B and C. The standards used were phosphorylase *b*, bovine serum albumin, ovalbumin, α -chymotrypsinogen, β -lactoglobulin, and lysozyme (sizes are indicated in kilodaltons).

12. 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.
13. Kim, S.-D., and J. Spizizen. 1985. Molecular cloning and expression of *Bacillus pasteurii* urease gene in *Escherichia coli*. *Korean J. Appl. Microbiol. Bioeng.* **13**:297–302.
14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
15. 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.
16. MacLaren, D. M. 1969. The significance of urease in *Proteus* pyelonephritis: a histological and biochemical study. *J. Pathol.* **97**:43–49.
17. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. Mobley, H. L. T., G. R. Chippendale, M. H. Fraiman, J. H. Tenney, and J. W. Warren. 1985. Variable phenotypes of *Providencia stuartii* due to plasmid-encoded traits. *J. Clin. Microbiol.* **22**:851–853.
19. 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.
20. Mobley, H. L. T., and J. W. Warren. 1987. Urease-positive bacteriuria and obstruction of long-term urinary catheters. *J. Clin. Microbiol.* **25**:2216–2217.
21. Rosenstein, I. J. M., and J. M. T. Hamilton-Miller. 1984. Inhibitors of urease as chemotherapeutic agents. *Crit. Rev. Microbiol.* **11**:1–12.
22. Rubin, R. H., N. E. Tolkoﬀ-Rubin, and R. S. Cotran. 1986. Urinary tract infection, pyelonephritis, and reflux neuropathy, p. 1085–1141. In B. M. Brenner and F. C. Rector, Jr. (ed.), *The kidney*, 3rd ed., vol. 2. The W. B. Saunders Co., New York.
23. Samtoy, B., and M. M. DeBeukelaer. 1980. Ammonia encephalopathy secondary to urinary tract infection with *Proteus mirabilis*. *Pediatrics* **15**:294–297.
24. Smith, C. J., R. B. Hespell, and M. P. Bryant. 1980. Ammonia assimilation and glutamate formation in the anaerobe *Selenomonas ruminantium*. *J. Bacteriol.* **141**:593–602.
25. Todd, M. J., and R. P. Hausinger. 1987. Purification and characterization of the nickel-containing multicomponent urease from *Klebsiella aerogenes*. *J. Biol. Chem.* **262**:5963–5967.
26. Warren, J. W. 1986. *Providencia stuartii*: a common cause of antibiotic-resistant bacteriuria in patients with long-term indwelling catheters. *Rev. Infect. Dis.* **8**:61–67.
27. Warren, J. W., J. H. Tenney, J. M. Hoopes, H. L. Muncie, and W. C. Anthony. 1982. A prospective microbiologic study of bacteriuria in patients with chronic indwelling urethral catheters. *J. Infect. Dis.* **146**:719–723.
28. Weatherburn, M. W. 1967. Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* **39**:971–974.
29. Wilkinson, G. N. 1961. Statistical estimations in enzyme kinetics. *Biochem. J.* **80**:324–332.