JOURNAL OF BACTERIOLOGY, Mar. 1988, p. 1027–1033
0021-9193/88/031027-07$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 170, No. 3

Multiple Proteins Encoded within the Urease Gene Complex of *Proteus mirabilis*

STEPHEN E. WALZ,* SUSAN K. WRAY, SHEILA I. HULL, AND RICHARD A. HULL

Department of Microbiology and Immunology, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77030

Received 22 June 1987/Accepted 30 November 1987

Chromosomal DNA fragments from a uropathogenic isolate of *Proteus mirabilis* were inserted into the cosmid vector pHC79 to construct a genomic library in *Escherichia coli* HB101. A urease-positive recombinant cosmid, designated pSKW1, was recovered. Sequential recombinant manipulation of pSKW1 yielded a 10.2-kilobase plasmid, designated pSKW4, which encoded three urease isozymes with electrophoretic mobilities identical to those of the donor *P. mirabilis* strain. Plasmid pSKW4 gene sequences encode seven proteins designated 68K (apparent molecular weight, of 68,000), 28K, 25K, 22.5K, 18.5K, 7.5K, and 5.2K within the limits of the urease gene complex. Insertion mutations in genes encoding the 68K, 28K, 25K, 22.5K, 7.5K, and 5.2K proteins resulted in complete or partial (22.5K) loss of urease activity. There was no reduction in urease activity when the gene encoding the 18.5K protein was inactivated.

Urease (urea amidohydrolase, EC 3.5.1.5), which was first isolated from jack beans (*Canavalia ensiformis*) by Sumner in 1926 (37), is widely distributed in the evolutionary hierarchy. It can be found in human tissues, molluscs, plants, molds, yeasts, and numerous species of bacteria (6, 28, 32) including *Proteus mirabilis*, which is the organism most commonly associated with urinary calculi in humans (28). The ability to hydrolyze urea with subsequent alkalinization of the urine and infectious stone formation is a virulence factor *P. mirabilis* shares with other uropathogenic representatives of the family *Enterobacteriaceae*, most notably members of the tribe *Proteeae*, and to a lesser extent *Klebsiella* spp. (2, 11, 21, 29). Historically, quantitative variation in urease production among the enteric organisms led to the development of biochemical media and tests which could distinguish the rapidly ureolytic *Proteus* spp. from those which hydrolyzed urea more slowly (certain *Klebsiella*, *Enterobacter*, *Citrobacter*, *Yersinia*, and *Serratia* spp.) (4, 7, 9, 36, 38). The inclusion of *Proteus morganii* (now *Morganella morganii*) in the genus *Proteus* on the basis of its rapid urease reaction was challenged by Guo and Liu (12), who were able to serologically distinguish the urease of *P. morganii* from that of the rest of the *Proteus* spp. Further evidence for the qualitatively distinct nature of bacterial ureases was provided by Senior et al. (33), who separated the ureases produced by *P. mirabilis*, *P. vulgaris*, and *P. rettgeri* from each other and from that of *P. morganii* by comparing the electrophoretic mobilities of their urease activity bands in nondenaturing polyacrylamide gels. These four classical *Proteus* spp. are currently represented by five species in three genera within the tribe *Proteeae* (*P. mirabilis*, *P. vulgaris*, *Providencia rettgeri*, *P. stuartii* urease positive, and *M. Morganii* [26]).

Jack bean urease has an estimated molecular weight of 590,000, consists of six identical subunits, and migrates as a single activity band in nondenaturing gels (1, 8, 34). Depending on the source, bacterial urease reportedly ranges from molecular weight 151,000 to 800,000 (5, 10, 30, 31; J. A. Anderson, F. Kopko, A. J. Siedler, and C. G. Nohle, Fed. Proc. **28**:764, 1969), may have nonidentical subunit composition (5, 14, 25), and migrates as two (*P. stuartii*), four (certain *P. rettgeri* strains), or in most cases three activity bands in nondenaturing gels (17, 25, 33). Hydroxamic acids are effective inhibitors of both plant and bacterial urease (13, 18).

Regulation of bacterial urease synthesis varies depending on the organism and test conditions (17, 22, 25, 29, 31). The ureases of *P. mirabilis*, *P. vulgaris*, *P. rettgeri*, and *P. stuartii* have been reported to be urea inducible (22, 25, 29). Zorn et al. (41), however, concluded that in *P. rettgeri* urease synthesis is controlled by repression through ammonia, and Rosenstein et al. (29) found urease to be constitutively produced by *M. morganii*. Acetohydroxamic acid, a potent urease inhibitor with therapeutic potential, facilitates urease induction in *P. mirabilis*, *P. vulgaris*, and *P. rettgeri* (29). Urease is reportedly a cytoplasmic enzyme in *Klebsiella aerogenes* (10) but is associated with the periplasm and outer membrane in *P. mirabilis* (24).

Since variability in product and regulation with regard to bacterial urease is primarily determined by the DNA sequences associated with its synthesis, the isolation of these sequences and identification of their associated products is a prerequisite to evaluating the role of urease as a virulence factor in urinary tract infections or in determining its utility as a taxonomic criterion. Mobley et al. (25) have previously reported the cloning of urease gene sequences from *P. stuartii*. In this study we describe the isolation of urease gene sequences from a uropathogenic strain of *P. mirabilis* and their expression in *Escherichia coli* HB101.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. mirabilis* HU1069 is a uropathogenic isolate obtained from the Ben Taub County Hospital, Houston, Tex. (40). *E. coli* HB101 (*pro leu thi lacY hsdS20 endA recA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44*) was the recipient for transformation with recombinant plasmids. *E. coli* HU736 is *E. coli* K-12 strain MV12 containing the plasmid Fts::Tn*5* lac^+ pro^+.

Bacteria were grown on L agar or in L broth without glucose (20) supplemented with ampicillin (100 μg/ml), kanamycin (20 μg/ml), and 0.1% urea as required.

The urease activity of bacterial transformants was determined on Christensen urea agar (4) supplemented with yeast

* Corresponding author.

extract (5 g/liter), tryptone (10 g/liter), and ampicillin (100 μg/ml).

Enzymes and buffers. Restriction endonucleases were obtained from Bethesda Research Laboratories (Gaithersburg, Md.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and New England Biolabs (Beverly, Mass.). Conditions and buffers used for digestion were those recommended by the manufacturers. Klenow enzyme was obtained from Boehringer Mannheim, and T4 DNA ligase was obtained from Bethesda Research Laboratories. Ligation buffer contained 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 10 μM dithiothreitol, and 40 μM ATP. TE buffer was 10 mM Tris (pH 8) and 1 mM EDTA.

Construction of recombinant molecules. High-molecular-weight chromosomal DNA was isolated from *P. mirabilis* HU1069 by the method of Hull et al. (15). DNA fragments ranging from 21 to 30 megadaltons were obtained by partial digestion with *Sau*3A followed by NaCl gradient centrifugation and ethanol precipitation. These fragments were suspended in ligation buffer with *Bam*HI-cut pHC79 DNA and ligated in the presence of T4 ligase for 48 h at 4°C (16). Recombinant molecules were packaged in vitro into bacteriophage λ using a commercial packaging kit (Bethesda Research Laboratories) and used to infect *E. coli* HB101. Transductants harboring recombinant plasmids were selected on L agar containing ampicillin and screened for urease production by replica plating on supplemented Christensen urea agar. Plasmid DNA was purified by the cleared lysate procedure (35). Deletion and subsequent recombination of isolated plasmid DNA were achieved by digestion with appropriate restriction endonucleases followed by ligation (as above) and transformation of competent *E. coli* HB101 (3).

Urease activity gels. Urease-producing bacteria were grown to the midlog phase in 500 ml of L broth supplemented with urea and harvested by centrifugation at 4°C. They were washed twice in 10 mM Tris (pH 7)–10 mM EDTA, suspended in 10 ml of the same, and ruptured by passage through a French pressure cell at 28,000 lb/in^2. Lysates were centrifuged at 12,000 × *g* for 5 min, and the supernatants were electrophoresed in a nondenaturing polyacrylamide gel (6% resolving gel; 4% stacking gel; 1:32, bisacrylamide/acrylamide) as described by Mobley et al. (25). After electrophoresis the gel was equilibrated by consecutive 30-min rinses in 1 mM EDTA (pH 5) containing 50 mM sodium acetate, 20 mM sodium acetate, and 0.02% cresol red, respectively. Urease activity bands were visualized by immersion in 1.5% urea–0.02% cresol red–1 mM EDTA. When crimson-colored activity bands became apparent they were cut out of the gel and immersed in 0.1 M lead acetate solution to terminate the reaction and preserve the bands as insoluable lead precipitate (34). The gel was reassembled and photographed by using electrophoresis development paper (Eastman Kodak Co., Rochester, N.Y.).

Purification of plasmid DNA fragments. Plasmid DNA was digested with restriction endonucleases. Reaction mixtures were diluted in several volumes of stop mix (5% Ficoll 400, 1.8% sodium dodecyl sulfate [SDS]), and fragments were separated by electrophoresis through 0.7% agarose (Bethesda Research Laboratories) gels with a Tris-borate-EDTA buffer system (23). Desired fragment bands were cut from the gel, and the DNA was eluted with an Elutrap electroseparation system (Schleicher & Schuell Co., Keene, N.H.). The purification process was repeated with GTG agarose (Sea Kem; FMC Corp., Rockland, Maine), and the DNA was alcohol precipitated and suspended in TE buffer.

In vitro translation of plasmid-encoded proteins. Circular plasmid or purified fragment DNA (1 to 4 μg) was transcribed and translated in vitro in the presence of L-[^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) for 45 min with a commercial procaryotic DNA-directed translation kit (Amersham Corp., Arlington Heights, Ill.) followed by a 5-min methionine chase. Translated proteins (10^5 trichloroacetic acid-precipitable cpm per lane) were solubilized at 100°C for 5 min in SDS-gel sample buffer and electrophoresed on SDS-polyacrylamide gels (15% resolving gel; 4% stacking gel; 1:32, bisacrylamide/acrylamide) by the method of Laemmli (19). After electrophoresis, gels were fixed in 30% methanol–10% glacial acetic acid, dried onto filter paper, and autoradiographed with XAR-5 film (Eastman Kodak Co., Rochester, N.Y.).

Transposon mutagenesis. *E. coli* HB101(pSKW4), a urease-encoding clone isolated in this study, was mixed with *E. coli* HU736 at an initial concentration of 10^7 CFU of each per ml in 20 ml of L broth without antibiotics and incubated overnight at 30°C without shaking. Transconjugants were selected by plating serial 10-fold dilutions of the mating culture on L agar containing ampicillin and kanamycin. Plates with approximately 500 well-isolated transconjugant colonies were harvested to 1 liter of L broth with ampicillin, and cultures grown to an optical density at 600 nm of 0.4. Plasmids were amplified overnight in the presence of chloramphenicol (170 μg/ml) and isolated by the method of So et al. (35). Purified plasmid was used to transform *E. coli* HB101 to ampicillin and kanamycin resistance. Transformants were tested for urease activity, and plasmid DNA was screened for Tn*5* insertion by observing its migration in 0.7% agarose gels relative to nonmutagenized pSKW4. The site and orientation of Tn*5* insertion were determined by examining restriction fragments generated during single digests of plasmid DNA with *Bam*HI, *Pst*I, and *Hin*dIII.

Site-directed mutagenesis. Plasmid pSKW4 was digested completely with *Bam*HI or partially with *Bst*EII, and full-length linear DNA was purified as described above. Protruding 5′ ends were filled by using Klenow enzyme in the presence of all four deoxynucleoside triphosphates by the method of Wartell and Reznikoff (39) and ligated with T4 DNA ligase for 16 h at 15°C. The ligation mixture was alcohol precipitated, suspended in TE buffer, and used to transform *E. coli* HB101 to ampicillin resistance. Transformants were tested for urease activity, and plasmid DNA was examined for loss of the target restriction site.

RESULTS

Isolation of recombinant plasmids encoding urease. Bacteriophage λ transducing particles carrying recombinant cosmid molecules with *P. mirabilis* HU1069 sequences were prepared and used to transduce *E. coli* HB101. Approximately 1% of the ampicillin-resistant transductants produced an alkaline reaction on supplemented Christensen urea agar, indicating urease activity. A 43-kilobase (kb) plasmid, designated pSKW1, was purified from one of the urease-positive clones. Complete digestion of pSKW1 with *Eco*RI followed by ligation and transformation of *E. coli* HB101 yielded a 30-kb urease-encoding plasmid, pSKW2, which was subsequently reduced to 17.4 kb by deletion of three *Nhe*I fragments. This 17.4-kb urease-encoding plasmid, designated pSKW3, was the source of insert DNA in the construction of pSKW4 (10.2 kb) and pSKW7 (8.6 kb) (Fig. 1). Plasmid pSKW3 was digested with *Nhe*I, *Nde*I, and *Eco*RI, and pBR322 was digested with *Nhe*I and *Nde*I. Fragments

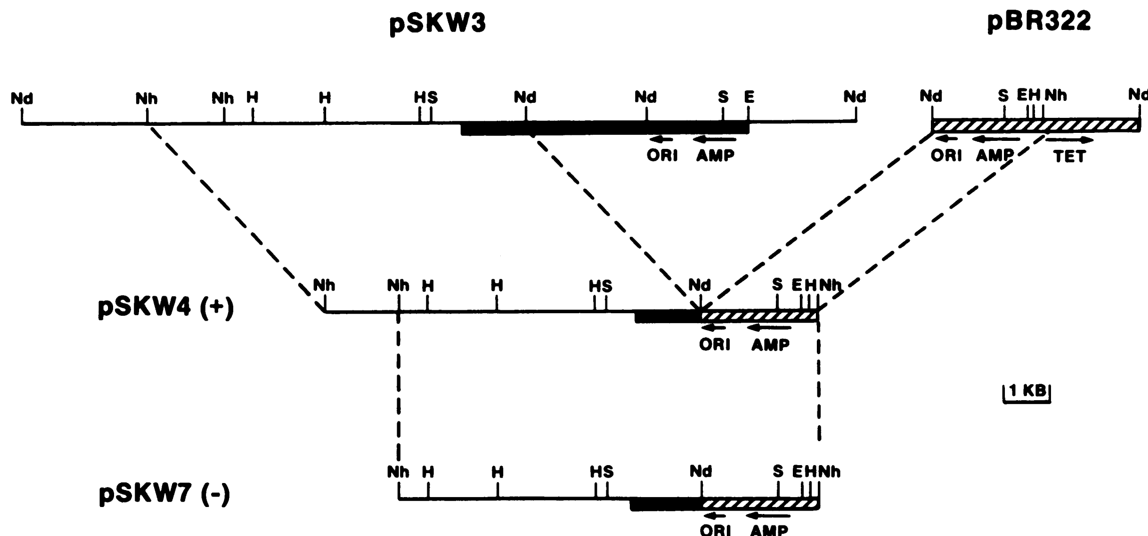

FIG. 1. Construction of recombinant plasmids pSKW4 and pSKW7. A triple restriction digest (*Eco*RI, *Nde*I, and *Nhe*I) of urease-encoding pSKW3 was ligated with a double digest (*Nde*I and *Nhe*I) of pBR322, and the mix was used to transform *E. coli* HB101 to ampicillin resistance. Recombinant plasmids pSKW4 and pSKW7 were recovered. As indicated, pSKW4 encodes urease (+) on *P. mirabilis* insert sequences (thin lines). It also contains vector sequences from pHC79 (thickened lines) and pBR322 (hatched lines). Plasmid pSKW7 does not contain the 1.6-kb *Nhe*I fragment present on pSKW4 and is urease negative (−). Restriction endonuclease recognition sites are designated as follows: E, *Eco*RI; H, *Hin*dIII; Nd, *Nde*I; Nh, *Nhe*I; and S, *Sca*I. Plasmid origins of replication (ORI) and antibiotic resistance genes (TET, tetracycline; AMP, ampicillin) are indicated.

from both digestions were mixed, ligated, and used to transform *E. coli* HB101 to ampicillin resistance. Restriction fragment analysis of plasmid DNA from the transformants demonstrated a urease-positive and a urease-negative population represented by pSKW4 and pSKW7, respectively. Both plasmids have the 6.3-kb *Nde*I-*Nhe*I fragment from pSKW3 containing 1.4 kb of sequence derived from pHC79. They differ in that pSKW4 has an additional 1.6 kb of insert DNA on an *Nhe*I-*Nhe*I fragment. All further attempts to reduce the size of the insert DNA by partial digestion with *Sau*3A and *Hpa*II resulted in loss of urease activity.

Urease activity gel. Senior et al. (33) reported that *P. mirabilis* urease migrates as three activity bands in nondenaturing polyacrylamide gels. To determine whether pSKW4 encoded all three urease isozymes, lysates of urea induced *P. mirabilis* HU1069 and *E. coli* HB101(pSKW4) were electrophoresed in nondenaturing polyacrylamide gels as described in Materials and Methods. Both lysates produced three activity bands with identical electrophoretic mobilities (Fig. 2), but the time of appearance and intensity of the bands differed. The fastest-migrating band (α-urease) in the *P. mirabilis* lysate developed within 1 min and was focused and intense. It was followed at equal distances by the β-urease and γ-urease bands. The β-urease developed more slowly than the α-urease and was less intense. Only after prolonged incubation (>5 min) did the γ-urease band of *P. mirabilis* become apparent. This situation was reversed for the *E. coli* HB101(pSKW4) lysate, in which the γ-urease band developed first, followed by the β-urease band and much later (>5 min) by the α-urease band. The slower-developing pSKW4-encoded urease bands were less intense than its γ-urease band, and none of these was as focused or intense as the α-urease band of *P. mirabilis* HU1069.

In vitro translation of urease gene sequences. Plasmid pSKW4 DNA was digested to completion with *Hin*dIII, and fragments of 1.5, 2.0, 2.4, and 4.3 kb were purified (Fig. 1). The 6.3-kb *Nde*I-*Nhe*I fragment was also purified. Fragment and undigested pSKW4 DNA were transcribed and translated in vitro and the denatured, radiolabeled proteins were electrophoresed in an SDS-polyacrylamide gel and autoradiographed (Fig. 3). The four pSKW4 *Hin*dIII fragments represent mutually exclusive coding regions comprising the entire plasmid. The major proteins encoded by these individual *Hin*dIII fragments were identified by the intensity of their corresponding autoradiographic bands (Fig. 3, lanes 2, 3, 4, and 5). The 2.4-kb *Hin*dIII fragment translation (lane 3) contained two major proteins, 18.5K and 25K (estimated molecular weights of 18,500 and 25,000, respectively). The 2.0-kb *Hin*dIII fragment translation (lane 4) contained three major proteins, 5.2K, 7.5K, and 28K. Numerous protein

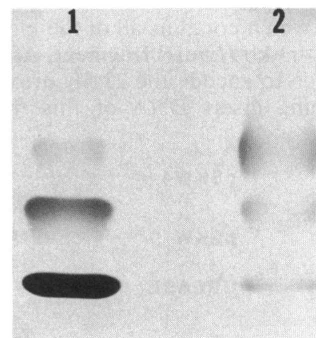

FIG. 2. Nondenaturing polyacrylamide gel electrophoresis of native and recombinant urease. Urease isozymes present in cell lysates of urea-induced *P. mirabilis* (lane 1) and *E. coli* HB101(pSKW4) (lane 2) are presented. Isozyme bands were visualized by immersion in a solution of urea, cresol red, and EDTA. As crimson-colored activity bands became apparent they were cut from the gel and immersed in 0.1 M lead acetate solution to terminate the reaction and preserve the bands as white lead precipitate. Gel pieces were reassembled for photography.

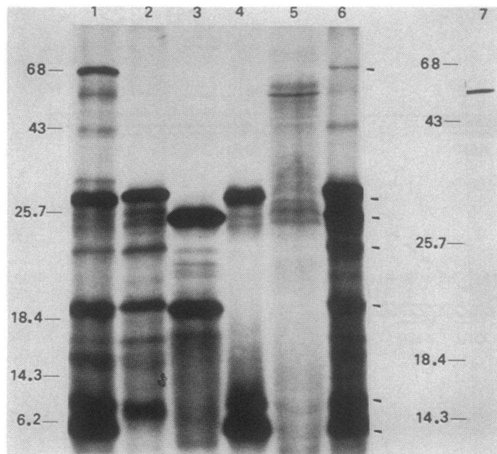

FIG. 3. Autoradiograph of [^{35}S]methionine-labeled proteins encoded on pSKW4. Restriction fragments and undigested pSKW4 DNA were translated in vitro by a coupled transcription-translation procedure. Radiolabeled proteins were denatured in SDS-gel sample buffer and electrophoresed in a 15% SDS-polyacrylamide gel. Lanes: 1, 6.3-kb *Nde*I-*Nhe*I fragment; 2, 4.3-kb *Hin*dIII fragment; 3, 2.4-kb *Hin*dIII fragment; 4, 2.0-kb *Hin*dIII fragment; 5, 1.5-kb *Hin*dIII fragment; 6, undigested pSKW4; and 7, negative DNA control. Major proteins encoded by *P. mirabilis* insert sequences are indicated opposite the undigested pSKW4 translation (lane 6). The positions of molecular size standards are given in kilodaltons.

bands of varied intensity were present in the 4.3-kb *Hin*dIII fragment translation (lane 2). Bands corresponding to 6.5K, 18.5K, and 30K proteins were judged to be clearly more intense than the other bands in this same lane and therefore considered to represent major fragment-encoded proteins. A band of intermediate intensity corresponding to a protein of approximately 22,500 molecular weight was also present in this 4.3-kb fragment translation. Since this protein was subsequently shown to be required for complete expression of urease activity (see below), it too was considered a major fragment-encoded protein. The 4.3-kb *Hin*dIII fragment consists mainly of pHC79 and pBR322 vector sequences. The 30K protein is beta-lactamase. The 6.5K protein must be encoded, at least in part, on pBR322 sequences since it is not synthesized from the 6.3-kb *Nde*I-*Nhe*I fragment (Fig. 3, lane 1 versus lane 2), which contains all of the insert and pHC79 sequences of the 4.3-kb *Hin*dIII fragment. After allowing for sufficient sequence to encode the 22.5K urease-related protein, the remaining insert DNA of this fragment is not sufficiently long to encode the 18.5K protein, which must therefore derive from pHC79 sequences. No major proteins bands were present in the 1.5-kb *Hin*dIII fragment translation (Fig. 3, lane 5). The band corresponding to a 57K protein in this translation is endogenous to the in vitro translation system. The same band can be seen in samples containing 2.5×10^4 cpm from in vitro translations incubated in the absence of added DNA (Fig. 3, lane 7) and has also been reported by Pratt et al. (27).

An additional major protein of 68K, encoded by pSKW4 but not present in any of the *Hin*dIII fragment translations, was evident in the 6.3-kb *Nde*I-*Nhe*I fragment translation (Fig. 3, lane 1). The seven proteins (68K, 28K, 25K, 22.5K, 18.5K, 7.5K, and 5.2K) which appear to be encoded by *P. mirabilis* HU1069 insert sequences are identified in the sample lane containing the in vitro translation products of undigested pSKW4 DNA (Fig. 3, lane 6). The distinction between major and background protein bands was less clear in this translation, particularly with regard to the 68K protein. For this reason vector- and insert-encoded proteins were identified from the genetically simpler fragment translations.

Insertional mutagenesis. Seven unique derivatives of pSKW4, containing Tn*5* insertions in *P. mirabilis* HU1069 sequences, were prepared as described in Materials and Methods. They were designated pSKW52, pSKW56, pSKW60, pSKW108, pSKW111, pSKW126, and pSKW128. The position of Tn*5* insertion for each of these derivatives, relative to restriction endonuclease recognition sites, is presented in Fig. 4. The in vitro translation products of pSKW4 were compared with those of its insertionally mutagenized derivatives in SDS-polyacrylamide gels (Fig. 5, lanes 1, 2, 3, 4, 5, 8, 9, and 10). In lanes 8, 9, and 10 the 30K beta-lactamase band has been eliminated by digesting the plasmids with *Sca*I before translation. In addition to those protein bands previously observed in plasmid fragment translations (Fig. 3), multiple transposon-encoded proteins are also evident in the translations of plasmids containing Tn*5* insertions (Fig. 5, lanes 2, 3, 4, 5, 9, and 10). The two previously identified 18.5K proteins are resolved in Fig. 5 as having apparent molecular weights of 18,400 and 18,500.

Plasmid pSKW52, which contains a Tn*5* insertion in the 4.3-kb *Hin*dIII fragment, does not encode the 6.5K or 22.5K protein (Fig. 5, lane 2). Of these only the 22.5K protein was previously determined to be encoded by insert DNA. The faint band remaining at the 22.5K position in this lane is interpreted as the background protein present in the 2.4-kb *Hin*dIII fragment translation (Fig. 3, lane 3). Although a band corresponding to the 18.4K protein is not apparent in

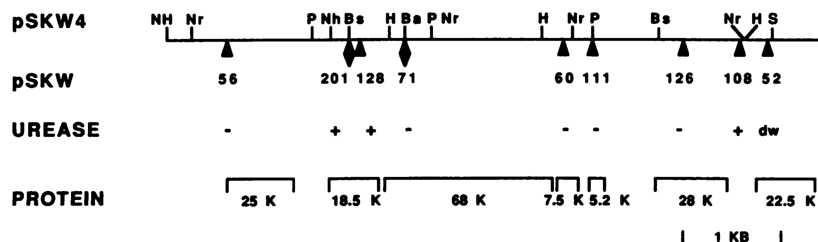

FIG. 4. Relative positions of insertion mutations of pSKW4 and proposed urease protein-coding regions. Symbols: ▲, transposon Tn*5* insertion mutations; ◆, restriction site-directed mutations. Only the insert region of pSKW4 is shown. Plasmid designations (pSKW number) and urease activity (+, positive; −, negative; and dw, delayed weak positive) are shown below their associated insertion sites. Proposed protein map positions relative to their coding region on pSKW4 are presented. The apparent molecular size in kilodaltons is indicated below each peptide. Restriction endonuclease recognition sites are designated as follows: Ba, *Bam*HI; Bs, *Bst*EII; H, *Hin*dIII; Nd, *Nde*I; Nh, *Nhe*I; Nr, *Nru*I; P, *Pst*I; and S, *Sca*I.

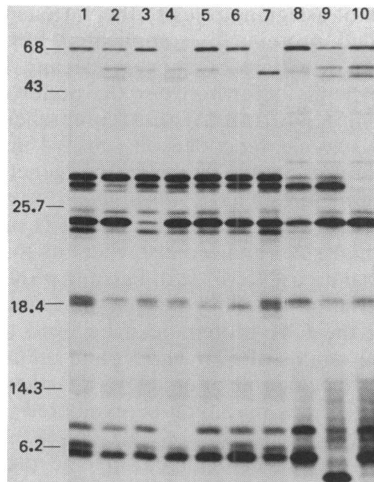

FIG. 5. Autoradiograph of [^{35}S]methionine-labeled proteins encoded on pSKW4 and its derivatives. Plasmid DNA was translated in vitro by a coupled transcription-translation procedure. Radiolabeled proteins were denatured in SDS-gel sample buffer and electrophoresed on 15% SDS-polyacrylamide gels. Lanes: 1, pSKW4; 2, pSKW52; 3, pSKW56; 4, pSKW60; 5, pSKW128; 6, pSKW201; 7, pSKW71; 8, pSKW4; 9, pSKW111; and 10, pSKW126. Plasmid DNA was digested with *ScaI* before translation for sample lanes 8, 9, and 10. The positions of molecular size standards are given in kilodaltons.

the autoradiograph of the pSKW52 translation products presented in Fig. 5, lane 2, a faint band at the 18.4K position was consistently present when longer exposure times were employed (data not shown). Synthesis of the 28K protein also appears reduced in the pSKW52 translation.

Transposon Tn*5* insertion in pSKW56 appears to affect synthesis of the 2.4-kb *Hin*dIII fragment-encoded 25K protein (Fig. 5, lane 3). The faint 25K band present in this translation may be transposon encoded or, alternatively, result from Tn*5* insertion in the promoter region for this protein. Plasmid pSKW7, which lacks the DNA region altered by Tn*5* insertion in pSKW56 due to a 1.6-kb *Nhe*I fragment deletion, also does not encode a 25K protein (Fig. 6).

Insert-encoded 7.5K and 18.5K proteins are absent in the translations of pSKW60 and pSKW128, respectively (Fig. 5, lanes 4 and 5). In the pSKW60 translation synthesis of the 68K protein is also reduced.

The 5.2K and 28K proteins are uniquely absent in translations of pSKW111 and pSKW126, respectively (Fig. 5, lanes 9 and 10). Because of prior digestion with *Sca*I, 30K, 22.5K, and 18.4K proteins are either faint or missing in the pSKW111 and pSKW126 translations. These protein bands are also either faint or missing in translations of *Sca*I-digested pSKW4 (Fig. 5, lane 8). *Sca*I recognizes two sites on these plasmids, one within the vector-coding region for beta-lactamase and the other on the insert (Fig. 1). The faint band which remains at the 30K position in Fig. 5, lanes 8, 9, and 10, seems to be encoded on the same region of DNA as the 28K protein and may represent an alternative form of this protein. Both the residual faint band at 30K and intense band at 28K are eliminated when the 2.0-kb *Hin*dIII fragment is digested with *Bst*EII before translation (Fig. 7, lane 1 versus lane 2).

Two additional derivatives of pSKW4, designated pSKW71 and pSKW201, were obtained by filling the 3′ recessed ends generated by digestion of pSKW4 with

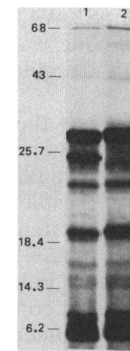

FIG. 6. Autoradiograph of [^{35}S]methionine-labeled proteins encoded on pSKW4 and pSKW7. Plasmids were translated in vitro using a coupled transcription-translation procedure. Radiolabeled proteins were denatured in SDS-gel sample buffer and electrophoresed in a 15% SDS-polyacrylamide gel. Lanes: 1, pSKW4; lane 2, pSKW7. The positions of molecular size standards are given in kilodaltons.

*Bam*HI and *Bst*EII, respectively. Plasmid pSKW71 encodes a truncated gene product of 55K in place of the complete 68K protein (Fig. 5, lane 7). This truncated protein is distinct from transposon-encoded proteins and diffuse background proteins of similar molecular weight. The pSKW201 translation did not contain the 18.5K protein, which was also the target of transposon insertion in pSKW128 (Fig. 5, lanes 5 and 6).

Urease activity and protein mapping. *P. mirabilis* HU1069 turned Christensen urea agar alkaline (urease positive) within 2 h. The urease reaction of *E. coli* HB101(pSKW4) on supplemented Christensen urea agar was positive within 6 h, whereas *E. coli* HB101(pSKW7) was still negative after 48 h of incubation. The urease reactions of the insertional mutations of pSKW4 are presented in Fig. 4. The sites of transposon insertion and proposed protein-coding regions, relative to restriction endonuclease recognition sites on pSKW4, are also indicated in Fig. 4. The proposed protein map positions were established from the pSKW4 fragment translations and from the translations of the insertionally

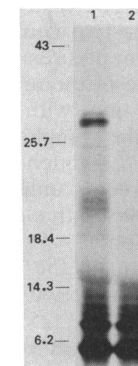

FIG. 7. Autoradiograph of [^{35}S]methionine-labeled proteins encoded on pSKW4 restriction fragments. A purified 2.0-kb *Hin*dIII fragment of pSKW4 was digested to completion with *Bst*EII and translated in vitro by a coupled transcription-translation procedure. Radiolabeled proteins were denatured in SDS-gel sample buffer and electrophoresed in a 15% SDS-polyacrylamide gel. Lanes 1, undigested 2.0-kb *Hin*dIII fragment; lane 2, 2.0-kb *Hin*dIII fragment after complete digestion with *Bst*EII. The positions of molecular size standards are given in kilodaltons.

mutagenized derivatives of pSKW4. The average molecular weight of an amino acid residue was assumed to be 110 in calculating the length of DNA required to encode a specific protein.

Plasmid pSKW7, which lacks the 1.6-kb *Nhe*I fragment present on pSKW4 and does not encode the 25K protein, is urease negative. This provides additional evidence for the placement of this protein and establishes that the left side limit of urease gene sequences extends to or near the end of the insert DNA. *E. coli* HB101(pSKW52) had a delayed (>8 h) and weak urease reaction, indicating that the 22.5K protein is part of the urease gene complex and extending the right side limit of urease gene sequences through the coding region for this protein. The 5.2K, 7.5K, 25K, 28K, and 68K proteins were absolutely required for urease activity. Clones unable to synthesize the 18.5K protein were urease positive. Plasmid pSKW108, which contains a Tn*5* insertion between the proposed coding regions for the 28K and 22.5K proteins, was urease positive and did not interrupt the synthesis of any of the identified proteins (data not shown).

DISCUSSION

An in vitro procaryotic DNA-directed translation system was used to identify urease gene complex proteins encoded by cloned fragments of *P. mirabilis* DNA. Although unidentified background proteins were present in these translations, major urease-related proteins were, with one noted exception, readily identified by the intensity of their autoradiographic bands. Synthesis of all identified urease-related proteins was subsequently interrupted by insertional mutagenesis of the urease-encoding plasmid pSKW4. A background 57K protein which was apparent in some plasmid translations was also present in the negative DNA control, indicating that it was endogenous to the translation system. The remaining background proteins presented as faint, often diffuse, bands of lower molecular weight than the largest identified urease-related protein. As such they may represent degradations of the larger proteins or the products of incomplete coupled transcription-translation. Their role, if any, in urea hydrolysis by *P. mirabilis* was not determined.

The results of this study indicate that the urease gene complex of *P. mirabilis* encodes a minimum of seven proteins over 6.5 kb of DNA. The span of this complex is fixed by the requirement for the two flanking proteins (25K and 22.5K) for full expression of urease activity and by our inability to reduce the size of the urease-encoding insert of pSKW4 by partial digestion with enzymes *Sau*3A and *Hpa*II. It is possible that the complex extends beyond these limits to include *P. mirabilis* sequences not contained in the pSKW4 insert, but this seems unlikely since pSKW4 encodes three urease isozymes with electrophoretic mobilities identical to those of the donor.

The absolute requirement of the 5.2K, 7.5K, 25K, 28K, and 68K proteins for urease activity is consistent with the observation of Mobley et al. (25) that 25.5K and 73K proteins were associated with urease activity in *P. stuartii* and with the reported 72K, 11K, and 9K subunit composition of *K. aerogenes* urease (14). Since the 18.5K and 22.5K proteins are not absolutely required for urease activity, their role may be regulatory. The delayed weak urease reaction of *E. coli* HB101(pSKW52) could result from an insertion mutation in a regulatory gene. The cloned gene sequences in pSKW4, however, encode all three urease isozymes, so it is possible that its mutagenized but urease-positive derivatives are unable to direct the synthesis of one or even two of the three. The task of assigning roles to the various urease gene complex-encoded proteins is complicated further by the observation that the time of appearance and intensity of urease activity bands differ between the donor *P. mirabilis* strain and *E. coli* HB101(pSKW4). A comprehensive examination of the isozymes encoded on pSKW4 and its mutagenized derivatives could establish the genetic basis of urease isozyme synthesis in *P. mirabilis*.

The 68K protein reported in this study is analagous to proteins of similar size reported elsewhere as major components of bacterial urease (5, 14, 25). Plasmid pSKW71, which contains a restriction site-directed insertion mutation in the gene encoding the 68K protein, can be used to construct urease-negative strains of *P. mirabilis* and related ureolytic pathogens. The specific role of urease as a virulence factor in urinary tract infections may then be evaluated in an animal model system.

In this study we have localized specific protein-coding regions, relative to restriction endonuclease recognition sites, within the urease gene complex of *P. mirabilis*. This sequence-to-product relationship can be exploited in the examination of other bacterial species by DNA hybridization in a manner similar to that recently described by Jones and Mobley (17) for *P. stuartii* urease gene sequences. By employing a battery of protein specific DNA probes the taxonomic significance of these proteins within the family *Enterobacteriaceae* may be established. Such probes might also prove useful in bacterial identification.

ACKNOWLEDGMENTS

This work was supported by a research fellowship to S.K.W. from the National Kidney Foundation and by Public Health Service research grant AI 18462 from the National Institutes of Health.

LITERATURE CITED

1. **Blattler, D. P., C. C. Contaxis, and F. J. Reithel.** 1967. Dissociation of urease by glycol and glycerol. Nature (London) **216**:274–275.
2. **Braude, A. I., and J. Siemienski.** 1960. Role of bacterial urease in experimental pyelonephritis. J. Bacteriol. **80**:171–179.
3. **Brown, M. G., A. Weston, J. R. Saunders, and G. O. Humphreys.** 1979. Transformation of *Escherichia coli* C600 by plasmid DNA at different phases of growth. FEMS Microbiol. Lett. **5**:219–222.
4. **Christensen, W. B.** 1946. Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. J. Bacteriol. **52**: 461–466.
5. **Christians, S., and H. Kaltwasser.** 1986. Nickel-content of urease from *Bacillus pasteurii*. Arch. Microbiol. **145**:51–55.
6. **Cicmanec, J. F., S. L. Helmers, and A. I. Evans.** 1980. Office practice survey of bacterial pathogens causing urinary tract infections. Urology **16**:274–276.
7. **Cook, G. T.** 1948. Urease and other biochemical reactions of the *Proteus* group. J. Pathol. Bacteriol. **60**:171–181.
8. **Dixon, N. E., J. A. Hinds, A. K. Fihelly, C. Gazzola, D. J. Winzor, R. L. Blakeley, and B. Zerner.** 1980. Jack bean urease (EC 3.5.1.5). IV. The molecular size and mechanism of inhibition by hydroxamic acids. Spectrophotometric titration of enzymes with reversible inhibitors. Can. J. Biochem. **58**: 1323–1334.
9. **Elek, S. D.** 1948. Rapid identification of *Proteus*. J. Pathol. Bacteriol. **60**:183–192.
10. **Friedrich, B., and B. Magasanik.** 1977. Urease of *Klebsiella aerogenes*: control of its synthesis by glutamine synthetase. J. Bacteriol. **131**:446–452.
11. **Griffith, D. P., D. M. Musher, and C. Itin.** 1976. Urease: the primary cause of infection-induced urinary stones. Invest. Urol. **13**:346–350.

12. **Guo, M. M. S., and P. V. Liu.** 1965. Serological specifities of ureases of *Proteus* species. J. Gen. Microbiol. **38**:417–422.
13. **Hase, J., and K. Kobashi.** 1967. Inhibition of *Proteus vulgaris* urease by hydroxamic acids. J. Biochem. **62**:293.
14. **Hausinger, R. P.** 1987. Nickel utilization by microorganisms. Microbiol. Rev. **51**:22–42.
15. **Hull, R., S. Bieler, S. Falkow, and S. Hull.** 1986. Chromosomal map positions of genes encoding P adhesins in uropathogenic *Escherichia coli*. Infect. Immun. **51**:693–695.
16. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33**:933–938.
17. **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.
18. **Kobashi, K., J. Hase, and K. Uehara.** 1962. Specific inhibition of urease by hydroxamic acids. Biochim. Biophys. Acta **65**:380.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Lennox, E.** 1955. Transduction of linked genetic characteristics of the host by bacteriophage P1. Virology **1**:190–206.
21. **MacLaren, D. M.** 1969. The significance of urease in *Proteus* pyelonephritis: a histological and biochemical study. J. Pathol. **97**:43–49.
22. **Magana-Plaza, I., and R. Ruiz-Herrera.** 1967. Mechanisms of regulation of urease biosynthesis in *Proteus rettgeri*. J. Bacteriol. **93**:1294–1301.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **McLean, J. C., K.-J. Cheng, W. D. Gould, J. C. Nickel, and J. W. Costerton.** 1986. Histochemical and biochemical urease localization in the periplasm and outer membrane of two *Proteus mirabilis* strains. Can. J. Microbiol. **32**:772–778.
25. **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.
26. **Penner, J. L.** 1984. *Proteus*, *Providencia*, and *Morganella*, p. 491–498. *In* Bergey's manual of systematic bacteriology. The Williams & Wilkins Co., Baltimore.
27. **Pratt, J. M., G. J. Boulnois, V. Darby, E. Orr, E. Wahle, and I. B. Holland.** 1981. Identification of gene products programmed by restriction endonuclease DNA fragments using an *E. coli in vitro* system. Nucleic Acids Res. **9**:4459–4474.
28. **Rosenstein, I. J., and J. M. Hamilton-Miller.** 1985. Inhibitors of urease as chemotherapeutic agents. Crit. Rev. Microbiol. **11**: 1–12.
29. **Rosenstein, I. J., J. M. Hamilton-Miller, and W. Brumfitt.** 1980. The effect of acetohydroxamic acid on the induction of bacterial ureases. Invest. Urol. **18**:112–114.
30. **Rosenstein, I. J., J. M. Hamilton-Miller, and W. Brumfitt.** 1981. Role of urease in formation of infectious stones: comparison of ureases from different sources. Infect. Immun. **32**:32–37.
31. **Schneider, J., and H. Kaltwasser.** 1984. Urease from *Arthrobacter oxydans*, a nickel containing enzyme. Arch. Microbiol. **139**:355–360.
32. **Seneca, H., P. Peer, and R. Nally.** 1962. Microbiol urease. Nature (London) **193**:1106–1107.
33. **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.
34. **Shaik-M, M. B., A. L. Guy, and S. K. Pancholy.** 1980. An improved method for the detection and preservation of urease activity in polyacrylamide gels. Anal. Biochem. **103**:140–143.
35. **So, M., W. S. Dallas, and S. Falkow.** 1978. Characterization of an *Escherichia coli* plasmid encoding for synthesis of heat-labile toxin: molecular cloning of the toxin determinant. Infect. Immun. **21**:405–411.
36. **Stuart, C. A., E. Van Stratum, and R. Rustigian.** 1945. Further studies on urease production by *Proteus* and related organisms. J. Bacteriol. **49**:437–444.
37. **Sumner, J. B.** 1926. The isolation and crystallization of the enzyme urease. J. Biol. Chem. **69**:435–441.
38. **Vuye, A., and J. Pijck.** 1973. Urease activity of *Enterobacteriaceae*: which medium to choose. Appl. Microbiol. **26**: 850–854.
39. **Wartell, R. S., and W. S. Reznikoff.** 1980. Cloning of DNA restriction endonuclease fragments with protruding, single-stranded ends. Gene **9**:307.
40. **Wray, S. K., S. I. Hull, R. G. Cook, J. Barrish, and R. A. Hull.** 1986. Identification and characterization of a uroepithelial cell adhesin from a uropathogenic isolate of *Proteus mirabilis*. Infect. Immun. **54**:43–49.
41. **Zorn, C., R. Dietrich, and H. Kaltwasser.** 1982. Regulation by repression of urease biosynthesis in *Proteus rettgeri*. Z. Allg. Mikrobiol. **22**:197–203.