

## Sequence of the *Klebsiella aerogenes* Urease Genes and Evidence for Accessory Proteins Facilitating Nickel Incorporation

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A 4.8-kilobase-pair region of cloned DNA encoding the genes of the *Klebsiella aerogenes* urease operon has been sequenced. Six closely spaced open reading frames were found: *ureA* (encoding a peptide of 11.1 kilodaltons [kDa]), *ureB* (11.7-kDa peptide), *ureC* (60.3-kDa peptide), *ureE* (17.6-kDa peptide), *ureF* (25.2-kDa peptide), and *ureG* (21.9-kDa peptide). Immediately after the *ureG* gene is a putative rho-dependent transcription terminator. The three subunits of the nickel-containing enzyme are encoded by *ureA*, *ureB*, and *ureC* based on protein structural studies and sequence homology to jack bean urease. Potential roles for *ureE*, *ureF*, and *ureG* were explored by deleting these accessory genes from the operon. The deletion mutant produced inactive urease, which was partially purified and found to have the same subunit stoichiometry and native size as the active enzyme but which contained no significant levels of nickel. The three accessory genes were able to activate apo-urease in vivo when they were cloned into a compatible expression vector and cotransformed into cells carrying the plasmid containing *ureA*, *ureB*, and *ureC*. Thus, one or more of the *ureE*, *ureF*, or *ureG* gene products are involved in nickel incorporation into urease.

Urease (EC 3.5.1.5) was the first enzyme to be crystallized (37) as well as the first shown to contain nickel (6). Early studies involving urease made use of enzyme purified from jack beans; this homohexameric plant enzyme has continued to be extensively studied, as reviewed by Andrews et al. (1). Recently, interest has focused on ureases from bacterial sources, which differ from the plant enzyme in subunit composition and native molecular weight (30). The urease of *Klebsiella aerogenes* (currently *K. pneumoniae*) is similar to those of several other bacteria; it contains four tightly bound nickel ions distributed among two active sites per native molecule and consists of three subunits in an  $\alpha_2\beta_4\gamma_4$  stoichiometry (39, 40). The genes for *K. aerogenes* urease were recently cloned, and the enzyme was overexpressed in *Escherichia coli* (33). Inactive urease apoenzyme was synthesized when recombinant cells were grown in the absence of nickel ion (33), ruling out nickel-dependent transcriptional regulation. Subsequent addition of nickel to whole cells led to apoenzyme activation, even after treatment with protein synthesis inhibitors (23). In contrast, purified apoenzyme could not be activated in vitro by the addition of nickel, indicating that some cellular component may be required for nickel incorporation into the enzyme (23).

In this report we present the DNA sequence of the six open reading frames in the *K. aerogenes* urease operon. Three of these genes are shown to encode the urease subunits. The precise role of the remaining three genes is unknown; however, evidence is presented that these accessory genes function in activating urease apoenzyme by incorporating nickel.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *E. coli* JM109 (43) and XL-1-B (2), grown at 37°C in 2xYT medium (28), served as hosts for the M13 clones used for sequencing. *E. coli* DH1 (14), used as the recipient for all studies involving urease expression, was grown at 37°C in morpholinepropanesulfon-

ic acid-glutamine medium supplemented with 100  $\mu$ M NiSO<sub>4</sub> and appropriate antibiotics as previously described (33).

**DNA sequencing.** All restriction enzyme digestions, end fillings, and other common DNA manipulations, unless otherwise stated, were performed by standard procedures (27, 34). Sequencing was carried out on a portion of the urease operon from the upstream *Sst*I site to the downstream *Hind*III site of plasmid pKAU17 (33) (Fig. 1). This fragment contains all of the urease operon genes but lacks urease activity due to partial deletion of the upstream promoter region. Portions of the *Sst*I-*Hind*III fragment were cloned into phage vector M13mp19 (43), and a series of unidirectional deletions was constructed for each strand by using exonuclease III and the method of Henikoff (15). Phage DNA from selected deletion clones was purified (28) and sequenced with Sequenase enzyme (U.S. Biochemical Corp., Cleveland, Ohio) and [<sup>35</sup>S]dATP as recommended by the manufacturer. Duplicate reaction sets were made for each clone: one with dGTP and the other with dTTP. Sequencing gels consisted of 6% acrylamide (19:1 acrylamide-bisacrylamide) and were either wedge shaped (0.4 to 1.2 mm) or straight (0.4 mm), in which case they were run by making the bottom buffer reservoir 1 M sodium acetate before electrophoresis (35). Sequence analysis was carried out for both strands. Alignments of overlapping sequence data were made by using the GENEPRO program (Riverside Scientific, Seattle, Wash.). Data base searches and open reading frame assignments were performed with the Wisconsin Genetic Computer Group software package version 6.1 (5). The Profilesearch program was used to look for homologous proteins in the National Biomedical Research Foundation sequence data base.

**Construction of plasmids pKAU601 and pKAU506.** Plasmid pKAU17 was partially digested with *Aat*I, the DNA fragments were electrophoresed in 1% agarose, and the band corresponding to a 1.5-kilobase-pair deletion was isolated by interception on DEAE paper (Whatman DE81). The DNA was eluted, ethanol precipitated, recircularized by T4 DNA ligase, and transformed into *E. coli* DH1. The resulting plasmid, pKAU520, was digested by several restriction

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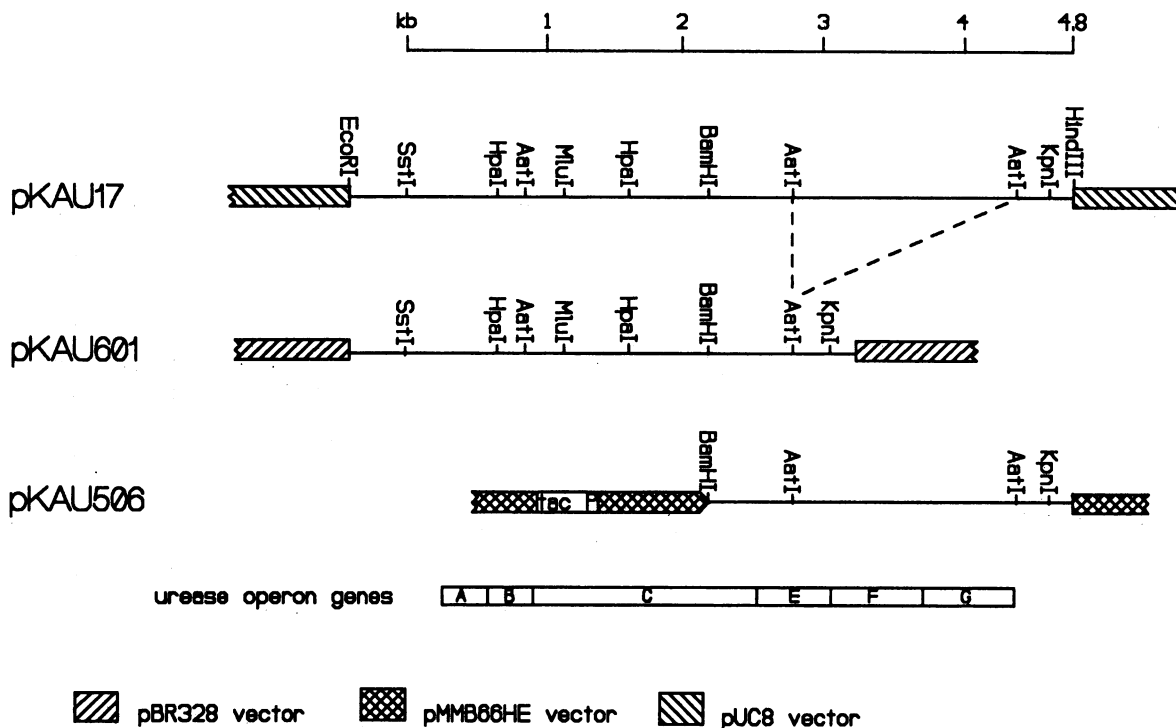


FIG. 1. Structure of the urease operon and two subclones. The restriction map for pKAU17, containing the cloned urease operon, is shown along with maps of two subclones derived from this plasmid. As described in the text and indicated at the bottom of the figure, the complete operon is comprised of six genes. The first three genes encode the urease structural subunits, whereas the function of the last three accessory genes may be related to nickel incorporation into urease. The structural genes and urease promoter region were subcloned into the ampicillin resistance gene of vector pBR328, yielding pKAU601. Similarly, the accessory genes were cloned under control of the *tac* promoter of expression vector pMMB66EH to give pKAU506. kb, Kilobase pairs.

enzymes to verify the proper deletion. To take advantage of more appropriate antibiotic markers, the pKAU520 fragment was cleaved from the pUC8 vector by digestion with *EcoRI* and *HindIII*. After treatment with Klenow fragment of *E. coli* DNA polymerase to produce blunt ends, the fragment was ligated into *PstI*-cut and Klenow fragment-treated pBR328 (36) to yield pKAU601 (Fig. 1).

A second subclone was made by digesting pKAU2687 (33), a precursor to pKAU17, with *BamHI*. The resulting 2.9-kilobase-pair fragment, which contains the *ureE*, *ureF*, and *ureG* region, was isolated as described above and ligated into *BamHI*-cleaved pMMB66EH (10; obtained from Michael Bagdasarian, Michigan Biotechnology Institute). The desired recombinant plasmid was verified by restriction analysis and designated pKAU506 (Fig. 1).

**Assays.** Urease activity was measured by quantitating the rate of ammonia released from urea by formation of indophenol, which was monitored at 625 nm (42). The assay buffer consisted of 25 mM *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  $\mu$ mol of urea per min at 37°C under the assay conditions described above. When urease activity was determined in cultures, cells were disrupted as previously described (33). Protein content was determined by the method of Lowry et al. (24) with bovine serum albumin as the standard.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** All gels for protein analysis were prepared by using the buffers of Laemmli (21) and consisted of either a 15%

polyacrylamide (acrylamide-bisacrylamide, 32:1) gel or a 10 to 15% polyacrylamide gradient resolving gel with a 4.5% polyacrylamide stacking gel. Gels were stained with Coomassie brilliant blue (Sigma) and scanned with a Gilford Response spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 540 nm.

**Purification and characterization of pKAU601-derived urease.** A 2-liter stationary-phase culture of *E. coli* DH1 containing plasmid pKAU601 was grown as described above. The cells were collected by centrifugation, suspended in 80 ml of 20 mM phosphate-1 mM EDTA-1 mM  $\beta$ -mercaptoethanol (pH 7.0) containing 0.5 mM phenylmethylsulfonyl fluoride, and sonicated for 3 min at 50% duty and 30% power with a Branson sonifier (Branson Sonic Power Co., Danbury, Conn.) equipped with a 0.5-in. (ca. 1.25-cm)-diameter tip. Cell debris was removed by sedimentation at 100,000  $\times$  *g* for 60 min at 4°C. Further purification of the pKAU601-derived urease from the cell extracts made use of room-temperature chromatography on DEAE-Sepharose, Superose 6, and Mono-Q resins obtained from Pharmacia (Uppsala, Sweden) as previously described for urease isolated from cells grown in the absence of nickel ion (23). Because the urease from this strain was enzymatically inactive, its presence was assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by comparison to authentic urease enzyme. The nickel content of the urease preparation was assessed by using atomic absorption spectrometry as previously described (32).

**Nucleotide sequence accession number.** The sequences determined here have been deposited in GenBank under the accession number M36068.

## RESULTS AND DISCUSSION

**Sequence analysis of the urease operon.** The sequence of a 4.8-kilobase-pair region encoding the urease operon is shown in Fig. 2. Analysis of the sequence revealed six open reading frames that are designated *ureA*, *ureB*, *ureC*, *ureE*, *ureF*, and *ureG*. These genes are all transcribed in the same direction and are predicted to encode peptides of 101, 106, 567, 158, 224, and 205 amino acids with  $M_r$ s of 11,086, 11,695, 60,304, 17,558, 25,221, and 21,943, respectively. The very close spacing of the genes results in three cases where the ribosome-binding site for one gene overlaps the coding region of the previous gene. Furthermore, the end of *ureB* overlaps the beginning of *ureC* by 8 nucleotides. *K. aerogenes* urease is expressed under nitrogen-limited conditions by the global Ntr system (9, 26). Detailed characterization of the urease upstream regulatory region will be described separately (Y. Markowicz, S. B. Mulrooney, and R. P. Hausinger, unpublished observations). *Proteus mirabilis* contains an additional urease gene (*ureD*) located immediately upstream of *ureA* (20); the *ureD* gene is thought to function in urea induction, a mode of regulation that does not occur in *K. aerogenes*. A potential rho-dependent transcriptional termination site has been identified immediately after the *ureG* gene. In addition, the first 75 amino acids of another open reading frame were detected starting at base 4534. It is preceded by two potential NtrC-binding sites (positions 4388 through 4402 and 4407 through 4421) and an NtrA-binding site (positions 4483 through 4499), consistent with nitrogen-dependent regulation. The partial open reading frame was not homologous to any sequence in the DNA or protein data base and is not part of the urease operon.

**Homology comparisons.** The predicted sequences for the first three genes of the *K. aerogenes* urease operon display significant homology to the reported amino acid sequence of jack bean urease (38): UreA is 59% identical to residues 1 through 101, UreB is 52% identical to residues 132 through 237, and UreC is 60% identical to residues 271 through 840 of the plant protein. The amino-terminal protein sequence of the large subunit of *K. aerogenes* urease (30) confirmed the assignment of the *ureC* gene to this polypeptide. Furthermore, the gene sequences encoding the *K. aerogenes* urease subunits are 72, 71, and 58% identical to recently reported sequences encoding urease subunits from *P. mirabilis* (20), *Proteus vulgaris* (31), and *Helicobacter pylori* (3), respectively. Indeed, 42% of the *K. aerogenes* amino acid residues are present in all three bacterial ureases and the jack bean enzyme. No significant homology was detected between the urease structural genes and any nonurease sequences in the National Biomedical Research Foundation data bank. Gene fusion or gene disruption may have occurred during evolution to explain the single-subunit plant protein, the two-subunit *H. pylori* sequence, and the three-subunit ureases found in other bacteria.

The predicted sequences for UreE, UreF, and UreG displayed no homology to the amino acid sequence of jack bean urease. Furthermore, little homology was observed when these sequences were compared with other sequences in the National Biomedical Research Foundation data bank. By contrast, sequences determined for the *ureE* and *ureF* genes of the *P. mirabilis* urease operon (20) were 53 and 38% identical to the *K. aerogenes* genes. Moreover, the reported *P. mirabilis* sequence included the start of an open reading frame (located 60 nucleotides beyond the termination of *ureF*) that was homologous to *ureG* from *K. aerogenes*. In addition, the limited DNA sequence data reported for the

urease operons from *P. vulgaris* (31) and *H. pylori* (3) included regions corresponding to the start of *ureE*.

**Complementation analysis of the urease operon genes.** When a region extending from the middle of *ureE* to the end of *ureG* was deleted from the urease operon (plasmid pKAU601, Fig. 1), no activity could be detected in transformed cells (Table 1). Sodium dodecyl sulfate-polyacrylamide gel analysis showed that the *ureA*, *ureB*, and *ureC* gene products were being expressed, although at lower levels than those seen with the intact operon (results not shown). A fragment containing the missing *ureE*, *ureF*, and *ureG* genes was subcloned behind the *tac* promoter of the compatible expression vector pMMB66HE to yield pKAU506 (Fig. 1). Expression of the genes was verified by sodium dodecyl sulfate-polyacrylamide gel analysis; polypeptides corresponding to the predicted sizes for UreE (17 kilodaltons [kDa]), UreF (25 kDa), and UreG (22 kDa) were clearly seen upon induction with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (data not shown).

When plasmids pKAU506 and pKAU601 were cotransformed into the *E. coli* host, the cotransformant was ureolytic, and isopropyl- $\beta$ -D-thiogalactopyranoside enhanced urease activity 2.4-fold (Table 1). The high levels of urease activity in the uninduced controls may indicate that small amounts of the accessory gene products are able to activate large amounts of inactive urease. Thus, *ureE*, *ureF*, and *ureG* gene products could act in *trans* with *ureA*, *ureB*, and *ureC* to give active urease, and at least one of these accessory genes is required. In contrast, no activity was observed when sonicated cells containing pKAU601 were mixed with equal amounts of sonicated isopropyl- $\beta$ -D-thiogalactopyranoside-induced cells containing pKAU506. Inclusion of 1 mM ATP in the 20 mM phosphate-1 mM  $\beta$ -mercaptoethanol (pH 7.0) reaction buffer had no effect. Lack of urease activation under these conditions may indicate a requirement for intact membranes, a need for an energy source other than ATP, or a temporal requirement for the accessory proteins during urease folding.

A requirement for accessory genes has also been demonstrated for ureases from soybeans (29), the fungus *Aspergillus nidulans* (25), *Providencia stuartii* (32), *P. mirabilis* (19, 41), a urease-positive *E. coli* (4), *K. pneumoniae* (13), *P. vulgaris* (31), and *Staphylococcus saprophyticus* (11). Genetic studies of soybeans demonstrated the presence of two loci that are distinct from the embryo-specific or ubiquitous urease isozyme structural genes (29). Mutations in these loci result in the production of inactive urease protein, consistent with a possible role in maturation. *A. nidulans* has been shown to have four loci involved in urea utilization: *ureA*, encoding a urea permease; *ureB*, encoding urease; *ureC* of unknown function; and *ureD*, which has been suggested to participate in the synthesis or incorporation of a nickel cofactor (25). In the bacterial cases, transposon insertion mutants or deletion mutants downstream of the urease structural genes produced all three urease subunits but possessed little or no urease activity (4, 11, 13, 19, 31, 32, 41). Several of these mutants were defective in nonurease subunit peptides, which may correspond to one or more of the accessory genes described here. Furthermore, the *P. mirabilis* urease operon included sequences that are analogous to *ureE*, *ureF*, and the start of *ureG* (20). A deletion of *ureG* and the very end of *ureF* led to substantial losses of activity in the *P. mirabilis* clone.

**Purification and characterization of pKAU601-derived urease.** Although plasmid pKAU601 contained the normal promoter region and the three urease subunit genes, cells

1 CTCTCGCGAAGCTCCCTGGGTGGGCACCTTTGCTGTGCTATCGGGTACCGGATGCCCTGC  
 61 TCGACGGGGTGGCGGACGGCTGGCGCCGCTCGGTCTCTACGCCGGCCACGCTGACCG  
 121 ACCGCTGCTGACGGTGGCTTTCCTCAGTGACGATAATCTGATTGGCCAGCGGGTATGC  
 181 GCGAGTATGGCAGTTTCTGGCCCTCATCTCACGGTAAATCTCCCGTACTTCCCGCAA  
 241 TCTGGCTGACTTAAGAGAAGCTTATGGAAGTACCCCCGAGAAAAAGACAAGCTGTTC  
 M E L T P R E K D K L L L  
 301 TGTTCACGCCGCGCTGGTGGCGGAGCGTGGCTGGCCGCGGCCTGAAGCTCAACTATC  
 F T A A L V A E R R L A R G L K L N Y P  
 361 CGGAGTCCGTGGCCCTGATCAGCGCCTTTATTATGGAAGGCGCTGGGACGGCAAAAGG  
 E S V A L I S A F I M E G A R D G K S V  
 421 TGGCTCGCTGATGGAGGAAGCGCTCAGTCTGACCCGCGAGCAGGTGATGGAGGGC  
 A S L M E E G R H V L T R E Q V M E G V  
 481 TCCCGAAATGATCCCGGATATCCAGGTGAAGCCCTTCCCGACGGCTCGAAGCTGG  
 P E M I P D I Q V E A T F P D G S K L V  
 541 TCACCGTTCACAACCCGATTATCTGAGTAGCGCCATGATCCCGGTGAATATCAGTTA  
 T V H N P I I \* M I P G E Y H V K  
 601 AGCCCGTACAGATAGCCCTGAATACCGCGCGGCAACCTGTGCGCTGGTGGTGGAGAACC  
 P G Q I A L N T G R A T C R V V V E N H  
 661 ACGGCGATCGCCGATTCAVGTGGTCCGACTACCATTTCCCGGAGTTAAACCGCGCG  
 G D R P I Q V G S H Y H F A E V N P A L  
 721 TGAAGTTCAGCGTCAAGCGCGCGGCTATCGCTGAATATCCCGCGGCGCACGGCGG  
 K F D R Q Q A A G Y R L N I P A G T A V  
 781 TACGCTTGAACCCGCGCAAAACCGGAGGTGAGCTGGTGGCTTCCCGGTACCCCGG  
 R F E P G Q K R E V E L V A F A G H R A  
 841 CCGTCTCGGCTTCCCGCGGAGGTATGGGCCCTTGGAGGTAAACGATGAGTAATATT  
 V F G F R G E V M G P L E V N D E \*  
 M S N I  
 901 TCACCCAGGCTATGCCGATATGTTCCGCCACCCTGCGCGACAAGGTGCGCCTGGCA  
 S R Q A Y A D M F G P T V G D K V R L A  
 961 GATACCGAGCTGTGGATCGAGGTGGAGGACGATTTGACCACCTACGGGGAAGAGTCAAA  
 D T E L W I E V E D D L T T Y G E E V K  
 1021 TTCGGCGCGGCAAGTATCCCGACGGCATGGCGAGGACAGATGCTGGCCGCGGAC  
 F G G G K V I R D G M G Q G Q M L A R G  
 1081 TGTGTGACCTGTGCTCACCAACCGTGTGATCGTCTGACTGCGGGATCGTTAAGGCC  
 C V D L V L T N A L I V D H W G I V K A  
 1141 GATATCGCGTGAAGGACGGCCGATCTTCGCCATCGGCAAGCCGCGCAACCCGACATC  
 D I G V K D G R I F A I G K A G N P D I  
 1201 CAGCCCAAGTCCATCCCATCGCGCTGCGACGGAAGTATCGCCGCGAAGGAAAA  
 Q P N V T I P I G A A T E V I A A E G K  
 1261 ATTGTACCGCGCGGGATCGATACCCATATCACTGGATCTGTCGCGAGCAGCGGAA  
 I V T A G G I D T H I H W I C P Q Q A E  
 1321 GAGCGCTGCTCTGGCGTACCACCATGTTGCGCGCGGCAACCGCCGCGCGCGGCGG  
 E A L V S G V T T M V G G G T G P A A G  
 1381 ACCCATGCCACCCTGCACCCCGGCGCGTGGTATATCTCACGATGCTGCGAGCGGCC  
 T H A T T C T P G P W Y I S R M L Q A A  
 1441 GACAGCTGCGCGTCAATATCGCCTGCTGGGCAAGGAAACGTTTCTCAGCCGATGCC  
 D S L P V N I G L L G K G N V S Q P D A  
 1501 CTGCGGAGCAGGTGGCGGAGCGTATTGCGCTGAAGATCCATGAGGATGGGCGCC  
 L R E Q V A A G V I G L K I H E D W A G  
 1561 ACCCGCGCGGATGACTGTGCGTTAACCGTTCGCGGATGAAATGGACATCCAGTCCGC  
 T P A A I D C A L T V A D E M D I Q V A  
 1621 CTGCACAGGACACCTGAAATGAATCCGTTTGTGGAAGACACCTCGCGCCATCGGC  
 L H S D T L N E S G F V E D T L A A I G  
 1681 GGGCGCACATCCACACTTCCATACCGAAGGGCGCGCGCGCATGCGCGGACATC  
 G R T I H T F H T E G A G G G H A P D I  
 1741 ATCACCCTGCGCCACCCGAACATTTGCGCTGCTCCACCAACCCCAACGCTGCCCTAC  
 I T A C A H P N I L P S S T N P T L P Y  
 1801 ACCCTCAACACCATCGATGAACATCTCGATATGCTGATGGTCTGCCACCATCTGGACCG  
 T L N T I D E H L D M L M V C H H L D P  
 1861 GACATCGCGGAGGACGTGGCCTTTGCCGAGTCCGCGCATTCCGCGGAAACCATCGCTCG  
 D I A E D V A F A E S R I R R E T I A A  
 1921 GAAGAGTGTGCAGATCTCGCGCCTTCTCGCTCACCTCTCCGATTCCGAGGCCATG  
 E D V L H D L G A F S L T S S D S Q A M  
 1981 GGCCGCTCGGGAAAGTATTCTCCGACCTGGCAGGTGGCGCATCGCATGAAGGTGCG  
 G R V G E V I L R T W Q V A H R M K V Q  
 2041 CGCGGAGCGTGGCGGAGGACCGGGGATAACGACAACTCCGCGTGAAGCGCTACATC  
 R G A L A E E T G D N D N F R V K R Y I  
 2101 GCCAAATACCATCAACCCGCGCTGACCCACGGCATCGCACAGAGTCCGATCCATT  
 A K Y T I N P A L T H G I A H E V G S I  
 2161 GAGTGGGTAAAGTGGTGCCTCGTGGTCTGGTCCAGGCTCTTCCGCGTGAACCG  
 E V G K L A D L V V W S P A F F G V K P  
 2221 GCCACCGTATCAAAGCGCGATGATCGCCATCGCCGATGGCGGATCAATGCCCTCT  
 A T V I K G G M I A I A P M G D I N A S  
 2281 ATTCGACCCCGCAGCGGTGCACTACCGCCGATGTTGGCGCGTGGGACGCGCCCG  
 I P T P Q P V H Y R P M F G A L G S A R  
 2341 CATCACTGCGCCTCACTTCTGTGCGAGCGCGGCGACGCAATGGCGTTCGCGAGCGG  
 H H C R L T F L S Q A A A A N G V A E R  
 2401 CTGAACCTGCGCAGCGGATCGCGTGGTGAAGGCTGGCTACGGTGCAGAAAGCCGAC  
 L N L R S A I A V V K G C R T V Q K A D  
 2461 ATGGTGCACAACAGTTCGACGCTAACATACCGTTCGAGCCGACGACCTGAGGTGCGG  
 M V H N S L Q P N I T V D A Q T Y E V R  
 2521 GTGGATGGCAACTTATCACCAGGAGCGCGAGAGTCTGCGGATGGCGCAACGATAT  
 V D G E L I T S E P A D V L P M A Q R Y  
 2581 TTTCTGTTTTAAGAGACCGGATGCTTTATTAACCTCAACGCTGAGATCCCGCGCC  
 F L F \* M L Y L T Q R L E I P A A  
 2641 GCGACCGCACGCTTACGCTGCCGATTGATGTTCCGCTCAAAGCCGGGTTAAGTCCAC  
 A T A S V T L P I D V R V K S R V K V T  
 2701 CTCAACGATGGCGGGATGCGCGCTGCTGCTCCCGCGCGCTGCTACTAOCGCGCGG  
 L N D G R D A G L L L P R G L L L R G G  
 2761 GATGTGCTCAGCAACGAAGAGCACCGAGTTTGTGAGGTGATGCGCTGATGAAGAG  
 D V L S N E E G T E F V Q V I A A D E E  
 2821 GTGTCGTTAGTGCCTGCGCAGTCCGTTTATGCTGGCAAGGCGCTGCTACCCACCTCGG  
 V S V V R C D D P F M L A K A C Y H L G  
 2881 AACCGTCACTGCGCTGAGATCATCGCGGCGAGCTGCGCTACCATCAGATCAGTG  
 N R H V P L Q I M P G E L R Y H H D H V  
 2941 CTGACGATATGCTGCGCAGTTCGCGCTGACGCTGACCTTTGGCCAGCTGCGGTTGAG  
 L D D M L R Q F G L T V T F G Q L P F E  
 3001 CCGGAAGCCGCGCTTACGCGAGGAGCCACGGTATCATCATGCTCATGACCCAC  
 P E A G A Y A S E S H G H H A H H D H  
 3061 CACGCTCACAGCCACTAGCATGTCGACAGCGGAACAACGCTGCGGCTGATGACGCTGG  
 H A H S H \* M S T A E Q R L R L M Q L A  
 3121 CAGCAGCAACTCGCGGTAGGGGTTACAGTGGTCCAGGGGCTGGATGGCTGTGGA  
 S S N L P V G G Y S W S Q G L E W A V E  
 3181 AGCCGCTGGGTGCGAGCTGCGCGCTTCGAGCGCTGGCAGCGGACGCGGATGACGGA  
 A G G W L D V A A F E R W Q R R Q M T E  
 3241 AGGCTTTTTTACCCTGACCTGCGCTGTTGCGCCGCTGTACCGCGCTGCGAACAAGG  
 G F F T V D L P L F A R L Y R A C E Q G  
 3301 CGATATGCTGCGCGCCAGCGCTGGACCGCTATCTGCTGGCCTGCGCGGAAACTCGTGA  
 D I A A A Q R W T A Y L L A C R E T R E  
 3361 ACTCGGGAGGAAGAGCGCAACCGCGCGCGGCTTTGCGGCTGCTGAGCGACTGGCA  
 L R E E E R N R G A A F A R L L S D W Q

FIG. 2. Nucleotide sequence of the urease genes. The deduced amino acid sequences for the six open reading frames are shown for *ureA* (base pairs [bp] 264 through 566), *ureB* (bp 576 through 896), *ureC* (bp 889 through 2592), *ureE* (bp 2602 through 3078), *ureF* (bp 3080 through 3754), and *ureG* (bp 3763 through 4380). Putative Shine-Dalgarno sites are underlined, and a possible rho-dependent transcription termination sequence is indicated by arrows.

3421 GCCGGACTGTCGCGCCGCGTGGCGCTCCCTGTGCCAGCAAAGCCAGCTCGCCGGGATGGC  
 P D C P P P W R S L C Q Q S Q L A G M A

3481 CTGGCTCGGCGTGGCTGGCGTATCGCCCTGCCGAGATGCCCTCAGCTGGGCTATAG  
 W L G V R W R I A L P E M A L S L G Y S

3541 CTGGATTGAGAGCCGCGTATGGCCGGCGTCAAGCTGGTCCCTTCGGCCAGCAGGCCGC  
 W I E S A V M A G V K L V P F G Q Q A A

3601 CCAGCAGCTGATTTTACGCTTTGTGACCACTACGCGCCGAGATGCCCGCGCGCTGGC  
 Q Q L I L R L C D H Y A A E M P R A L A

3661 CGCGCCGGAGCGGATATCGGATCGGCCACCCGCTCGCCGCATCGCTCTGCCGGCA  
 A P D G D I G S A T P L A A I A S A R H

3721 TGAAACCAATACTCTCGATTATTCGTTCTTAGGAGAAGCCATGAACTTTATAAACAC  
 E T Q Y S R L F R S \* M N S Y K H

3781 CGCTGCGCGTGGCGTGGCGGCCGCTCGGTAACCCGCTCTGCTGGAAGCG  
 P L R V G V G G P V G S G K T A L L E A

3841 CTGTGTAAGGATGCGCGATACCTGGCAGTGGCGGTGGTCACTAACGACATCTATACC  
 L C K A M R D T W Q L A V V T N D I Y T

3901 AAAGAAGATCAGCGCATCTCCACCAAGCGGGCGCTGGCGCCTGAACGCATCGTCGGT  
 K E D Q R I L T E A G A L A P E R I V G

3961 GTGGAAACCGGGCGTCCCGCATACCGCGATCCCGAAGATGCCTCAATGAACCTCGCC  
 V E T G G C P H T A I R E D A S M N L A

4021 GCGGTGAAGCGTGAAGTTCGGTAACCTCGACCTTATCTCGTGGAAAGCGGC  
 A V E A L S E K F G N L D L I F V E S G

4081 GCGATAACCTGAGCGCCACCTTCAGCCCGGAGTGGCGGATCTGACCATCTACGTATC  
 G D N L S A T F S P E L A D L T I Y V I

4141 GATGTGGCCGAGGGGAGAAGATCCCGCGCAAAGCGGACCGGGATCACAAATCCGAT  
 D V A E G E K I P R K G G P G I T K S D

4201 TTCCTGGTATCAATAAAACCGACCTTCCCGCCATGTGGCGCGTCCGCTGGAGGTGATG  
 F L V I N K T D L A P Y V G A S L E V M

4261 GCGAGCGATACCCAGCGTATGCCGCGCATGCCCATGGACCTTACCAATCTGAAGCAG  
 A S D T Q R M R G D R P W T F T N L K Q

4321 GCGACGGCTGAGCACCATTATCGCCTTCTCGAAGACAAAGGCATGCTGGCAAAATAG  
 G D G L S T I I A F L E D K G M L G K \*

4381 GCCTGTGACACCAGCGGGCGCAAAGCGGTCGGATGGTGACGCTCTGCTCTCTTTA

4441 TATCATCTGCTCCACCTCCGCGCCACGCTGCCCTGCAATATGGCATAAGGTTTGCTA

4501 ATTCAAGTCATGCCTAACCATTAAGGAATGACTATGTATCACTGGATCTTAACCCCTGAA

4561 TTACCCGCGACAACCGGACTTCCCGTACCCGGAAACCTTAGAAGATTACACCTTACGT

4621 TACGCCCGCTGAGCTTCCCGCGTGGGGTCCGGGCGTCTCGCGTACCCGCGCTGGC

4681 GGCATCGCTATCTGGCCGACTTTTCCATCGGCGCCAGCATCGGATGGCTGGGGCAC

4741 AGCAACGCCATCTATTGATC 4761

FIG. 2—Continued

containing this plasmid had no urease activity. To assess the role of the missing genes in this construct, inactive urease protein was purified by procedures that were nearly identical to that used for the native enzyme. Because no activity was present, the presence of urease protein in column fractions was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The intensity of bands on the gels demonstrated that cell extracts contained greatly decreased levels of urease protein compared with that found in cells containing pKAU17. Moreover, the amount of urease protein purified from this clone (approximately 1.5 mg) was less than 5% of that typically observed in cells containing pKAU17.

The final preparation of pKAU601-derived urease was only 51% homogeneous as estimated by densitometric analysis of Coomassie blue-stained sodium dodecyl sulfate-

TABLE 1. Specific activities of ureases from recombinant *E. coli* cultures

Culture	Urease sp act <sup>a</sup> (μmol of urea per min per mg)	
	-IPTG	+IPTG
<i>E. coli</i> DH1(pKAU506)	<0.2	<0.2
<i>E. coli</i> DH1(pKAU601)	<0.06	<0.06
<i>E. coli</i> DH1(pKAU506, pKAU601)	12.9	31.2

<sup>a</sup> For comparison, *E. coli* DH1(pKAU17) typically has a specific activity of 120 μmol of urea per min per mg.

polyacrylamide gels; nevertheless, several properties of the protein were characterized. Although differing somewhat from the predicted sizes, the three urease subunits were identical in size to that found in native enzyme (apparent  $M_r$ s, 9,000, 11,000, and 72,000). Moreover, they clearly remained associated throughout the purification, which included ion-exchange and gel-filtration chromatography. Gel scanning demonstrated that the ratio of 60.3-kDa to 11.7-kDa to 11.1-kDa subunits was 1.1:1.8:1.7, nearly identical to that observed in the native enzyme (39). Furthermore, the chromatographic properties of the pKAU601-derived urease matched that of the native enzyme, indicating a similar size [(60-kDa subunit)<sub>2</sub>(12-kDa subunit)<sub>4</sub>(11-kDa subunit)<sub>4</sub>] and charge. The only observed distinction from the native urease was in the nickel content. Whereas native enzyme was shown to possess 4 mol of nickel per mol of enzyme (39), the pKAU601-derived protein had less than 0.25 mol of nickel per mol of enzyme. Thus, one or more of the accessory gene products are involved in facilitating assimilation of nickel into apourease.

One possible role for the accessory genes involves nickel transport into the cell. However, we feel that this cannot be the only role for the accessory genes because nickel can enter *E. coli* cells via the magnesium transport system (17). Since our cells were grown in a medium containing 0.1 mM nickel, it seems probable that some nickel would enter the cell, yet we could not detect any measurable activity.

A second possible role for the accessory genes involves nickel incorporation into apourease. We had previously reported that *K. aerogenes* cells containing the recombinant urease plasmid pKAU19 grown in nickel-free medium synthesized urease apoenzyme and that the apoenzyme was activated upon addition of nickel even after the cells were treated with protein synthesis inhibitors (23). The purified apourease could not be reactivated by the addition of nickel, indicating that nickel incorporation does not occur by passive binding. It was proposed that some additional component was necessary to facilitate insertion of nickel into the apoenzyme. The accessory genes may participate in this process. In this regard, the carboxyl-terminal sequence predicted for UreE is particularly interesting: 10 of the last 15 amino acids are histidine residues. Histidine-rich regions are involved in metal binding sites for other proteins (e.g., the zinc-binding protein from albacore tuna plasma [7] and the copper-containing hemocyanin [12]); thus, such a sequence may bind nickel ions that are subsequently transferred to apourease. The only other known function for a histidine-rich region involves a 16-amino-acid peptide containing 7 adjacent histidyl residues; this sequence participates in regulation of the *Salmonella* histidine operon (18).

In summary, the formation of active urease requires activation by accessory proteins that function in nickel incorporation. Urease is not the only metalloenzyme that

requires accessory proteins for metal incorporation. For example, multiple gene products are required for proper incorporation of molybdenum into several enzymes, as reviewed by Hinton and Dean (16). Moreover, evidence has been presented for a copper insertase required for N<sub>2</sub>O reductase biosynthesis (22). There is also evidence that in vivo nickel incorporation into *Rhodospirillum rubrum* carbon monoxide dehydrogenase may require nickel processing (8). Further characterization of the accessory proteins involved in activating urease may aid in elucidating some of the general incorporation mechanisms for metalloproteins. Future efforts will be directed at characterizing how the *ureE*, *ureF*, and *ureG* gene products are involved in this process.

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