

## Organization of *Ureaplasma urealyticum* Urease Gene Cluster and Expression in a Suppressor Strain of *Escherichia coli*

OLIVIER NEYROLLES,\* STÉPHANE FERRIS, NILANE BEHBAHANI,  
LUC MONTAGNIER, AND ALAIN BLANCHARD

Unité d'Oncologie Virale, Institut Pasteur, 75724 Paris Cedex 15, France

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*Ureaplasma urealyticum* is a pathogenic ureolytic mollicute which colonizes the urogenital tracts of humans. A genetic polymorphism between the two biotypes of *U. urealyticum* at the level of the urease genes was found. The urease gene cluster from a biotype 1 representative of *U. urealyticum* (serotype 1) was cloned and sequenced. Seven genes were found, with *ureA*, *ureB*, and *ureC* encoding the structural subunits and *ureE*, *ureF*, *ureG*, and a truncated *ureD* gene encoding accessory proteins. Urease expression was not obtained when the plasmid containing these genes was incorporated into an opal suppressor strain of *Escherichia coli*, although this enzymatic activity was found in the same *E. coli* strain transformed with pC6b, a plasmid with previously cloned urease genes from the *U. urealyticum* T960 strain of biotype 2 (serotype 8). Although there are 12 TGA triplets encoding tryptophan within urease genes, the level of expression obtained was comparable to the levels reported for other bacterial genes expressed in *E. coli*. Nested deletion experiments allowed us to demonstrate that *ureD* is necessary for urease activity whereas another open reading frame located downstream is not. The promoter for *ureA* and possibly other urease genes was identified for both serotypes.

Members of the class *Mollicutes* (trivial name, mycoplasmas) are wall-less prokaryotes that have been characterized as the smallest self-replicating organisms (34). In humans, mycoplasmas colonize mucosal surfaces and identified pathogens include species from the genera *Mycoplasma* and *Ureaplasma*.

The 14 serotypes of *Ureaplasma urealyticum* that colonize the human species can be clustered into two biotypes (36). This species has been implicated in various diseases including urethritis, septic arthritis, urinary stone formation, and various infections of premature babies and pregnant women (7-9). It seems that strains from the two biotypes could have different pathogenic potentials, but an association between a specific disease and a biotype remains to be demonstrated (20, 31, 37).

The genus *Ureaplasma* is differentiated from the class *Mollicutes* by the production of urease (urea amidohydrolase), which hydrolyzes urea into ammonia and carbamic acid. In the presence of water, carbamic acid is cleaved to ammonia and carbonic acid, which results in an increase in pH. *U. urealyticum* lacks the conventional pathways for ATP production (glycolysis and arginine breakdown) which are present in the other species of mycoplasmas. Growth is inhibited by specific urease inhibitors and is dependent on urea (24), indicating that there is a major role for urease in the metabolism of ureaplasmas. It has also been demonstrated that urea hydrolysis is coupled to ATP synthesis (38) and generates an ammonium ion transmembrane gradient that could be used to activate an ATP synthetase, F<sub>1</sub>F<sub>0</sub> ATPase (40). In addition to this key role in ureaplasma metabolism, urease also contributes to the pathogenic potential of the ureaplasmas.

The increase of extracellular pH associated with urea hydrolysis has indeed been found to be important for the pathogenicity of urogenital bacteria. It was also shown that the inoculation of human urine with *U. urealyticum* induced the formation of struvite and carbonate-apatite, which are mineral

components of urinary stones (43). This result suggests that ureaplasma urease may be a factor of virulence, as has been demonstrated for the urease of *Proteus mirabilis* (22).

Three structural subunits of the ureaplasma purified enzyme were characterized as 72-kDa (UreC), 14-kDa (UreB), and 11-kDa (UreA) polypeptides (44). The native form of the ureaplasma enzyme is believed to be a hexamer with equimolar ratios of the three subunits. It was also shown that the 72-kDa subunit, UreC, is linked to nickel ions which are essential for urease activity (44).

The urease genes of *U. urealyticum* are chromosomal. A 7.6-kbp *EcoRI* DNA fragment from the *U. urealyticum* serotype 8 (biotype 2) chromosome was cloned in *Escherichia coli* and was shown to exhibit genetic homology with cloned urease genes from *Helicobacter pylori*, *Providencia stuartii*, and *E. coli* (4). Partial sequencing of this 7.6-kbp DNA fragment revealed three open reading frames (ORFs) encoding the urease structural subunits (3). Comparison of the deduced polypeptides with the published sequences of other bacterial ureases showed that the enzymes possess highly conserved sequences (for reviews, see references 11 and 29).

We report here, using Southern hybridization, the detection of urease genes from *U. urealyticum* serotypes 1, 3, and 6 (biotype 1) and serotypes 2 and 8 (biotype 2). The entire DNA fragment containing the urease genes from serotype 1 was sequenced, showing that the organization of these genes in *U. urealyticum* is not exactly the same as that in other bacterial species. Furthermore, we have obtained urease expression of genes from serotype 8 using an opal suppressor strain of *E. coli* which allowed us to determine the minimal genetic information required for efficient urease synthesis.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *U. urealyticum* 23 (serotype 2), 58 (serotype 4), 354 (serotype 5), co (serotype 7), T960 (type strain; serotype 8), Vancouver (serotype 9), western (serotype 10), k2 (serotype 11), U24 (serotype 12), U38 (serotype 13), 7 (serotype 1), 27 (serotype 3), Pi (serotype 6), and U26 (serotype 14) were serologically characterized by J. Robertson (Edmonton, Canada) and kindly provided by C. Bébéar (Bordeaux, France). Ureaplasmas were cultivated in modified SP4 (45) medium containing 1% urea (wt/vol) with an

\* Corresponding author. Mailing address: Unité d'Oncologie Virale, Institut Pasteur, 28, rue du Dr. Roux, 75724 Paris Cedex 15, France. Phone: (33-1)-45-68-89-06. Fax: (33-1)-40-61-34-65. Electronic mail address: neyrolle@pasteur.fr.

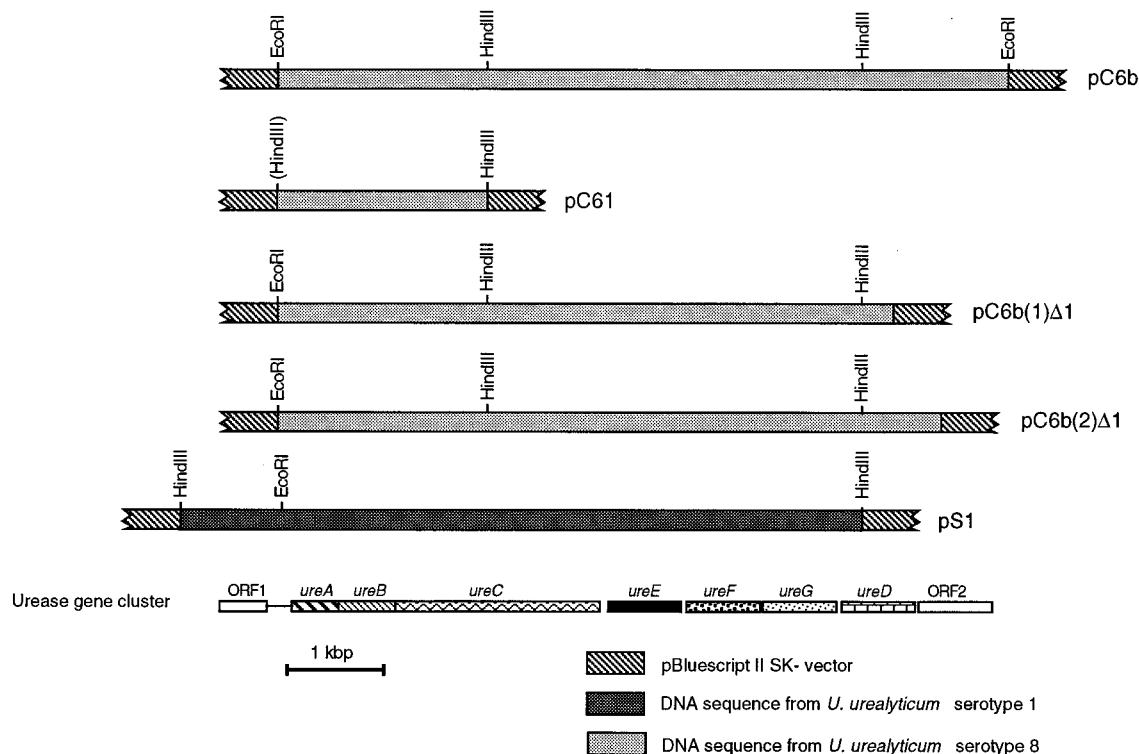


FIG. 1. Schematic representation of plasmids used in this study. The pC6b and pC61 plasmids were previously described (3, 4). The other plasmids were obtained during this study. The organization of the urease gene cluster is indicated, showing the genetic content of each plasmid.

initial pH adjusted to 5.5. After 60 h of incubation at 37°C, the *U. urealyticum* culture was centrifuged (1 h, 12,000 × g, 4°C), and collected cells were subsequently washed in phosphate-buffered saline (PBS) (KH<sub>2</sub>PO<sub>4</sub> [0.25 g/liter], K<sub>2</sub>HPO<sub>4</sub> [1.82 g/liter], NaCl [9 g/liter]). The pellet was resuspended in 1 ml of PBS, and the cellular suspension was stored at -70°C until use.

*Escherichia coli* XL1 Blue (Stratagene, La Jolla, Calif.) (*supE44 hsdR17 recA1 endA1 gyrA46 thi lac*), DH5α (GIBCO BRL, Life Technologies Inc., Gaithersburg, Md.) [*sup44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*], and PR101 (*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 mcrB1 mcrA Δlac-proAB recA [F' traD36 proAB<sup>+</sup> lac<sup>R</sup> ZΔM15]*) were transformed by recombinant plasmids. The PR101 strain of *E. coli* was obtained by introducing a *recA* mutation by transduction of the P1 virus in the ER1451 strain of *E. coli* (35). This strain was kindly provided by P. Renbaum (Jerusalem, Israel). These strains were cultivated in Luria broth or M9 minimal medium (39).

**Plasmids.** The pBluescript II SK- (Stratagene) plasmid was used as a cloning vector. To construct pSU77, the *trpT176* gene (*opal tRNA* suppressor under the control of an isopropylthio-β-D-galactoside [IPTG]-induced *lacUV5* promoter) was introduced into the pAL249 plasmid, derived from the pACYC184 plasmid (35). The pSU77 plasmid was kindly provided by P. Renbaum (Jerusalem, Israel). To construct pC6b, the previously described 7.6-kbp DNA fragment, IC6 (4), containing the urease genes from serotype 8 of *U. urealyticum* was subcloned at the *EcoRI* site of pBluescript II SK- (Fig. 1). To construct pC61, a 2.4-kbp *HindIII* DNA fragment (IC61), containing *ureA*, *ureB*, and truncated *ureC* genes (4), was subcloned at the *HindIII* site of pBluescript II SK- (Fig. 1).

**DNA extraction, digestion, and hybridization.** The pelleted *U. urealyticum* cells were lysed as previously described (4) for DNA extraction. The yield of extraction was approximately 10 μg of DNA per liter of culture.

Restriction enzymes and DNA-modifying enzymes were used according to the manufacturers' recommendations (Pharmacia Biotech [Uppsala, Sweden] and Boehringer Mannheim GmbH [Mannheim, Germany]).

The DNA fragments were separated by electrophoresis on a 0.8% agarose gel and transferred onto a nylon membrane (Hybond N+; Amersham Int. plc, Amersham, United Kingdom) by the method of Sambrook et al. (39). The membrane was incubated at 50°C for 1 h in the prehybridization solution (39). The DNA probe was labelled with [α-<sup>32</sup>P]dCTP by nick translation (nick translation kit from Amersham Int. plc). The hybridization was performed at 50°C for 15 h. The membrane was then washed, with the final wash being performed with 0.1 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.5% sodium dodecyl sulfate for 30 min at 55°C. DNA hybridization was revealed by autoradiography with Hyperfilm-MP (Amersham Int. plc).

**Purification of DNA from agarose gels and cloning of DNA.** After electro-

phoresis, DNA fragments were recovered by electroelution (39). These fragments were purified by chromatography on NACS prepac columns (GIBCO-BRL) and subsequently ligated to the appropriate vector by standard procedures (39). After transformation, plasmids were purified from selected *E. coli* clones by using Qiaprep kits (Qiagen Inc., Chatsworth, Calif.), following the manufacturer's protocol.

Nested deletions of plasmids were obtained by using exonuclease III with the Erase-a-Base kit (Promega Corp., Madison, Wis.). The pC6b plasmid was linearized by either *SacI* and *NotI* or *SacI* and *SmaI*. After exonuclease III digestion, DNA digests ranging in size from 5.0 and 7.2 kbp were selected. The resulting plasmids were recircularized and transformed into *E. coli* DH5α. Plasmids which were selected after size analysis were incorporated into *E. coli* PR101, and urease activity of resulting transformants was assessed in indole-urea indicator medium (see below).

PCR assays were performed by the method of Sambrook et al. (39).

**DNA sequencing and sequence analysis.** DNA sequencing was performed by using the Sequenase version 2.0 sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio). Both strands of DNA were sequenced.

Sequence analysis was performed by using the UNIX system, with the software package proposed by the Genetics Computer Group Inc. (Madison, Wis.) (15). The percentages of identity between urease polypeptides were calculated by using the Bestfit software with default values for alignment parameters.

**Primer extension experiments.** RNA from *U. urealyticum* was extracted by the method of Chomczynski and Sacchi (10). cDNA was synthesized by the standard protocol (39). The primers that were selected for these assays were 5'-AGCTA AACTGCTTCTTGAACGTCA-3' for serotype 1 (which corresponds to the reverse complementary sequence of nucleotides 982 to 1006 [see Fig. 3]) and 5'-ACCTTAGCTAAACGTCTTCTTGC-3' (which corresponds to the reverse complementary sequence of nucleotides 212 to 235 of the previously reported *ureA* sequence for serotype 8 [3]).

**Detection of ureolytic activity.** *E. coli* transformants were cultivated in 5 ml of M9 medium containing 100 μg of ampicillin per ml and 10 μM NiCl<sub>2</sub> at 37°C for 16 h. IPTG was added at a final concentration of 2.5 mM in order to induce the suppressor system, and the cultures were further incubated at 37°C for 4 h.

For the qualitative assay, 1 ml of culture was centrifuged (5 min, 12,000 × g, 4°C), and the cellular pellet was recovered in 1 ml of indole-urea indicator medium (Sanofi Diagnostic Pasteur, Marne la Coquette, France). The release of ammonia caused by urease activity increased the pH, inducing a color change from orange to red.

For the quantitative assay, urease activity was quantitated by the Berthelot reaction by a modified version of the procedure described by Ferrero and Lee

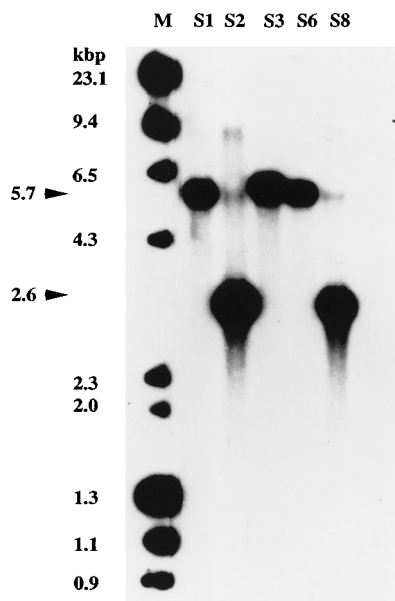


FIG. 2. Restriction fragment length polymorphism among the *Hind*III profiles of urease genes from the two *U. urealyticum* biotypes. Genomic DNAs from *U. urealyticum* biotype 1 (serotype 1 [lane S1], serotype 3 [lane S3], and serotype 6 [lane S6]) and biotype 2 (serotype 2 [lane S2] and serotype 8 [lane S8]) were digested with *Hind*III and electrophoresed. After transfer to a membrane, DNA hybridization was performed with the IC61 DNA fragment (containing the urease genes from serotype 8) as the probe. DNA markers ( $\lambda$  DNA digested by *Hind*III and  $\phi$ X174 DNA digested by *Hae*III) (Pharmacia Biotech) are shown in lane M.

(19) with *E. coli* cells in the mid-exponential phase of growth. The quantity of ammonia liberated was determined from a standard curve correlating  $A_{625}$  to the ammonium concentration (from  $\text{NH}_4\text{Cl}$ ). Urease activity was expressed as micromoles of  $\text{NH}_3$  per minute per milligram of bacterial proteins, and each value was the mean of three values averaged together. The protein concentrations were determined with the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, Ill.) with bovine serum albumin as a standard.

**Nucleotide sequence accession numbers.** The nucleotide sequences reported in this study will appear in the DDBJ, EMBL, and GenBank nucleotide databases with the accession numbers L40489 (data from Fig. 3) and L40490 (data from Fig. 6).

## RESULTS

**Restriction fragment length polymorphism among urease genes from the two biotypes of *U. urealyticum*.** Genomic DNAs from serotypes 1, 2, 3, 6, and 8 of *U. urealyticum* were digested with *Hind*III. After electrophoretic separation, the fragments were transferred onto a membrane and the IC61 DNA fragment was used as probe; IC61 contains full-length *ureA* and *ureB* genes and truncated *ureC* gene from *U. urealyticum* serotype 8. The results showed that the urease genes of serotypes 1, 3, and 6 (biotype 1) were detected on a 5.7-kbp DNA fragment whereas those of serotypes 2 and 8 (biotype 2) were localized to a 2.4-kbp DNA fragment as shown in Fig. 2. These data indicated a genetic polymorphism between the two biotype strains at the level of urease genes.

**Characterization of the urease genes from the genomic DNA of *U. urealyticum* serotype 1 (biotype 1).** Genomic DNA from *U. urealyticum* serotype 1 was digested with *Hind*III, and the fragments were separated by electrophoresis on a 0.8% agarose gel. The fragments whose size was close to 5.7 kbp were purified and ligated to the vector pBluescript II SK<sup>-</sup>. Ligation products were transformed into *E. coli* DH5 $\alpha$ . The clones containing the 5.7-kbp DNA fragment were selected by hybridization of purified plasmids with the IC61 DNA fragment as a

probe. One of the selected plasmids (pS1 [Fig. 1]) was further characterized, and the entire nucleotide sequence of the DNA insert was determined (Fig. 3). Sequence analysis showed that genes encoding urease structural subunits are located within 2.5 kbp, exhibiting an organization very similar to that of the serotype 8 urease genes (3). In agreement with the results from Southern blotting experiments (Fig. 2), no *Hind*III site was found within *ureC*, in contrast to the *Hind*III site found within this gene from serotype 8 (3). The urease subunits appear to be encoded by three genes, *ureA* (nucleotides 932 to 1234), *ureB* (nucleotides 1283 to 1654), and *ureC* (nucleotides 1703 to 3496). The deduced polypeptides (UreA [11.2 kDa], UreB [13.2 kDa], and UreC [64.5 kDa]) showed a high degree of identity with the homologous polypeptides of the serotype 8 urease (95% identity for the *ureA* products, 85% for the *ureB* products, and 92% for the *ureC* products). As with the urease genes of serotype 8 (3), the UGA codons encode Trp. The three polypeptides of serotype 1 urease contain 8 Trp residues, 6 of which have the same position as in the urease genes of serotype 8 (one TGA triplet is located within the *ureB* gene and seven TGA triplets are located within the *ureC* gene).

Furthermore, other ORFs were located on both sides of the genes encoding the structural subunits (Fig. 3): one ORF located upstream of *ureA* was named ORF1 (nucleotides 136 to 465; deduced molecular mass, 12.9 kDa) and four ORFs located downstream from *ureC* were named *ureE* (nucleotides 3564 to 4007; deduced molecular mass, 17.3 kDa), *ureF* (nucleotides 3952 to 4785; deduced molecular mass, 32.3 kDa), *ureG* (nucleotides 4791 to 5408; deduced molecular mass, 20.8 kDa), and *ureD* (nucleotides 5421 to 5699; this gene is truncated at its 3' end) by analogy to the organization and nomenclature that were used for other bacterial species. The name *ureD* was assigned to this gene after consideration of the homology of its product with other bacterial UreD polypeptides (see Discussion). The direction of transcription for these genes is the same as for the structural genes.

Each of the *ureA*, *ureB*, *ureC*, *ureE*, and *ureG* genes and ORF1 are preceded by a putative ribosomal binding site. For *ureE*, there is a possible ATG start codon at position 3734, in which case the deduced polypeptide would appear truncated compared with the polypeptides encoded by other *ureE* genes. However, 38 codons upstream from this TGA triplet, there is a TTG triplet which is the more probable start codon, because the length of the deduced UreE polypeptide is similar to those of UreE polypeptides from other ureolytic bacteria (for a recent review, see reference 29).

An ATP- or GTP-binding motif was found at the amino-terminal end of the *ureG* product (residues G-15PVGAGKT-22) as was shown for other bacterial UreG proteins (25, 41).

Figure 4 shows the alignment of urease genes from *P. mirabilis* (23), *Klebsiella aerogenes* (25, 30), *H. pylori* (13), *Yersinia enterocolitica* (14), *Bacillus* sp. strain TB-90 (27), and *U. urealyticum*. The percentages of identity between the products of these genes are also indicated in this figure. The three structural polypeptides (UreA, UreB, and UreC) and the UreG polypeptide are particularly well conserved among the six genera (from 44.5 to 65.7% of identity between the structural subunits and at least 51.1% between the UreG polypeptides). In contrast, polypeptides UreE, UreF, UreD, and the putative product encoded by ORF1 show a lower degree of conservation (Fig. 4). The highest percentages of identity with *U. urealyticum* polypeptides were found with those of *Bacillus* sp. strain TB-90. This result is in agreement with the phylogenetic positions of the six bacterial species that were analyzed, *U. urealyticum* and *Bacillus* sp. strain TB-90 being the only two representatives of the gram-positive eubacterial phylum.

AAG CTT CAT CAA TAA TTT GTT TTT CAC GTT CTG TAT TTG TAT TAA TTC CCA  
9 18 27 36 45

AAG CAG TTT AAC TTG GAA AAC GAC CCA TTT TAA CAA AAT CAT AAA TTG ATG TAG CTT  
60 69 78 87 96 105

CTG GGA ATA CAG CAA GTT GGG GGA CMT ATG CTA AAT TTA AGC CTA ATT OCT TTG ACT  
117 126 135 144 153 162

Y R H E F Y N F E Y V N Q L F R I T  
TAT AGG CAT GAA TTT TTT TAT AAT TTT GAA TAT GTC AAT CAA TTG TTT GAA ATA ACT  
174 183 192 201 210 219

S L R S F V N I L T I E D H A F S N Q  
TCT TTA CGA TCT TTT GTA AAT ATA TTA ACA ATA GAA GAT CAT GCT TTT TCT AAT CAA  
231 240 249 258 267 276

R N G N N G L D I N L S L Y I I L P L  
AGA AAT GGT AAT AAT GGT CTT GAT ATC AAT TTA TCT CTA TAC ATT ATA TTA CCA CTT  
288 297 306 315 324 333

E G L L I R H N P L I N V D L P E P L  
GAA GGA TTA TTG ATA CGA CAC AAT OCT TTG AAT AAT GTG GAT TTA CCA GAA CCA TTA  
345 354 363 372 381 390

G P N T T T K S L R W I V A V R F L I  
GGC OCT AAT ACA ACA AAA CCA TCG TTG CGA TGA ATT GTT GGC GTT AGG TTT TTA ATA  
402 411 420 429 438 447

I I F F P  
ATC ATT TTT TTT CCG TAA CCT AAT GAA ACA TTA TTA AGT TTA AAA TTG GCT CAT TAG  
459 468 477 486 495 504

AAA TCA ATT TCA TCG TTT TTA AAT TCT TCA CTA TCT TCA TAG CTT AAA ATT TCA TTC  
516 525 534 543 552 561

ATA TCT ACA ACC TAA GGT GTT TGT GGT CTT CTT TTT GTA AAT CAT TCT TTT TTT TAA  
573 582 591 600 609 618

AAA AAC TCA TAT TTA CTC CCA AAA CTA AAA AAT TAA ATT ATA AGT TAG AAT TTC ATT  
630 639 648 657 666 675

TTA TCT ACA AGA AGT TTA TCT TGT ATT CCC ATA AAA AAA TAA AAT GAA CAA ATT TTT TTT  
687 696 705 714 723 732

AAT TTC ATC ATT AAA GCA TTT ATT ATT TTT TTT ATT AAT TCT CCA ATA AGA ATA  
744 753 762 771 780 789

ACA CAT TTT TTT ATT CTA TAT ATT TTA CAT TTA CCA AAA AAA AAC ACT TTT TTT TAA  
801 810 819 828 837 846

TTT TAG TCT ATT TTT TTG TTT TAA AAG CTA TAA ATA AAA TTG CAT TAT TAC TTA ATA  
858 867 876 885 894 903

TAC AAG ATA TAT TAG AGA TAA ATA A ATG AAT CTA TCA TTA AGA GAA GTC CAA AAA TTA  
916 925 934 943 952 961

L I T V A A D V A R R R L L A R G L K L  
TTG ATA ACA GTT OCT GCT GCA GTT GCA AGA AGA CCA CTT TTA CCA AGA GGT TTA AAA TTA  
973 982 991 1000 1009 1018

N Y S E A V A L I T D H V M E G A R D  
AAC TAT TCA GAA GCT GTT GCT TTA ATT ACT GAT CAT GTA ATG GAA GGG CCA AGA GAT  
1030 1039 1048 1057 1066 1075

G K L V A D L M Q S A R E V L R V D Q  
GGT AGT CTA GTT GCT GCA CTA ATG CAA TCT GCT GCT GAA GAA CTA CCA GCT GAT CAA  
1087 1096 1105 1114 1123 1132

V M E G V D T M V S I I Q V E V T F P  
GTT ATG GAA GGT GTA GAT ACA ATG GTT AGT ATA ATT CAA GGT GAA GGT ACT TTC OCT  
1144 1153 1162 1171 1180 1189

D G T K L V S V H D P I Y K  
GAT GCT ACT AAA CTA GTT TCT GTA CAC GAT CCA ATT TAC TAA TAA CAT TTA CAT TCG  
1201 1210 1219 1228 1237 1246

TAA AAT TTT TTT ATA AGA GGA GAT AAT GAT TAT ATG TCA GGA TCA TCA AGT CAA TTT  
1258 1267 1276 1285 1294 1303

S P G K L V P G A I N F A S G E I V M  
AGT CCA GGT AAA TTA GTA CCA GGG CCA ATT AAT TTC GCT AGT GGT GAA ATT CTG ATG  
1315 1324 1333 1342 1351 1360

N E G R E A K V I S I K N T G D R P I  
AAT GAA GGT AGA GAG GCA AAA GTA ATT AGT ATT AAA AAT ACT GGG GAC GGT OCT ATA  
1372 1381 1390 1399 1408 1417

Q V G S H F H L F E V N S A L V F F D  
CAA GTT GGA TCA CAT TTT CAC TTG TTT GAA GTG AAT AGT CCA TTA GTA TTT TTT GAT  
1429 1438 1447 1456 1465 1474

E K G N E D K E R K V A Y G R R F D I  
GAA AAA GGA AAT GAA GAT AAA GAA CCA AAT GGT TAT GGA CCA CCA CCA GCT TTC GAT AAT  
1486 1495 1504 1513 1522 1531

P S G T A I R F E P G D K K E V S I I  
CCA TCA GGT ACT GCT ATT GGT TTT GAA CCA GCA GAT AAA AAA GAA GGT TCA ATT AAT  
1543 1552 1561 1570 1579 1588

D L A G T R E V W G V N G L V N G K L  
GAT TTA GCC GGA ACA CCA GAA GTT TGA GGT GTA AAT GGC TTA GTT AAT GGA AAA CTT  
1600 1609 1618 1627 1636 1645

K K . **UREC** M  
AAA AAA TAA AAC TCT ATT TTA CAA GTT TCT ACT ATA GAT AAA AAG GGG AAC ATT ATG  
1657 1666 1675 1684 1693 1702

F K I S R K N Y S D L Y G I T T G D S  
TTT AAA ATT TCA AGA AAA AAT TAT TCA GAT TTA TAC GGT ATT ACA ACT GGT GAT AGC  
1714 1723 1732 1741 1750 1759

V R L G D T N L W V K V E K D L T Y  
GTT AGA TTA GGA GAT ACA AAT CTT TGA GTT AAA GTT GAA AAA GAC TTA ACT ACT TAT  
1771 1780 1789 1798 1807 1816

G E E S V F G G G K T L R E G M G M N  
GGT GAA GAA TCT GTT TTT GGT GGT GCA AAA ACC CTA CCA GAT GAA GGT ATG GGA ATG AAT  
1828 1837 1846 1855 1864 1873

S T M K L D D K L G N A E V M D L V I  
TCT ACT ATG AAG TTA GAT GAT AAA TTA GGT AAT GCT GAA GAA ATA ATG GAT TTA GTT ATT  
1885 1894 1903 1912 1921 1930

T N A L I V D Y T G I Y K A D I G I K  
ACA AAT GCA CTA ATT GGT GAT TAT ACA GGT ATT GAT AAA GCA GAT AAT GCT ATT AAA  
1942 1951 1960 1969 1978 1987

N G K L A A I G K S G N P H L T D N V  
AAT GGA AAA CTT GCT GCG ATT GGA AAA TCT GGA AAT CCA CAT TTA ACA GAT AAT GGT  
1999 2008 2017 2026 2035 2044

D M I V G I S T E I S A G E G K I Y T  
GAT ATG ATT GTG GGT ATC TCA ACT GAA ATT TCA GCT GCT GGT GRG GGT AAA ATT TAT ACA  
2056 2065 2074 2083 2092 2101

A G G L D T H V H W L E P E I V P V A  
GCT GGT GGT TTA GAT ACT CAC GGT CAC TGA CTA GAA CCA GAA ATA GAT GGT GCT GGT CCA  
2113 2122 2131 2140 2149 2158

L D G G I T V I A G G T G M N D G T  
TTA GAT GGT GGT ATT ACA ACT GTT ATT GCT GGT GGT GGT ACA GGT ATG AAT GAT GGT ACA  
2170 2179 2188 2197 2206 2215

K A T T V S P G K F W V K S A L Q A A  
AAA GCC ACA ACT GTT TCA CCA GGT AAA TTT TGA GTT AAA TCA CCT TTA CAA CCG GCT  
2227 2236 2245 2254 2263 2272

D G L S I N A G F L A K G Q G M E D P  
GAT GGA TTA TCA ATT AAT GCT GGT TTT TTA GCT AAA GGT CAA GGT ATG GAA GAT CCA  
2284 2293 2302 2311 2320 2329

I F E Q I A A G A C G L K I H E D W G  
ATT TTT GAG CAA ATT GCT GCT GGA GCT TGT GGA CTT AAA ATC CAT GAA GAT TGA GGG  
2341 2350 2359 2368 2377 2386

A T G N A I D L A L T V A D K K D V A  
GCA ACA GGA AAT GCG ATT GAT TTA GCA TTA ACA GGT GCT GAT AAG ACT GAT GTA GCT  
2398 2407 2416 2425 2434 2443

V A I H T D T L N E A G F V E H T I A  
GTT GCT ATT CAT ACA GAT ACA TTA AAT GAA GCT GGA TTT GTA GAA CAT ACA ATT CCA  
2455 2464 2473 2482 2491 2500

A M K G R T I H A Y H T E G A G G G H  
GCT ATG AAA GGG CCA ACA ATT CAT GCT TAT CAT ACA GAA GGT OCT GGT GGA GCA CAT  
2512 2521 2530 2539 2548 2557

A P D I L E T V K Y A H I L P A S T N  
AAA GCC GAT ATT CTA GAA ACT GGT AAA TAT GCC CAT ATT TTA CCA OCT TCT ACA AAC  
2569 2578 2587 2596 2605 2614

P T I P Y T V N T I A E H L D M L M V  
CCA ACA ATT OCT TAT ACA GTA AAT ACA ATT GCT GAA CAT TTA GAT ATG TTA ATG GTA  
2626 2635 2644 2653 2662 2671

C H H L N P K V F E D V A F A D S R I  
TGT CAC CAC TTA AAT OCT AAG GTT CAA GAT GGT GCT TTT GCT GAT TCA CCA GCT ATT  
2683 2692 2701 2710 2719 2728

R S Q T I A A E D L L A D M G A I S I  
OCT AGC CAA ACA ATT GCA GGT GAA GAC TTA TTC GCC CAT TTA TTA GGT GAT GAT CCA ATT  
2740 2749 2758 2767 2776 2785

M S D T L A M C R I G E V A T R T W  
ATG TCA TCA GAT ACA TTA GCT ATG GGA GGT ATT GCT GGA GGT GCA ACT OCT ACA TGA  
2797 2806 2815 2824 2833 2842

Q M A H K M K A Q F G S L K G D S E F  
CAA ATG GCT CAC AAA ATG AAA CCA CAA TTT GGA TCA TTA AAA GGT GAT AGT GAA TTC  
2854 2863 2872 2881 2890 2899

S D N N R V K R Y I S K Y T I N P A I  
AGT GAT AAC AAT GGT GTA AAG CCT TAT ATT TCT AAA TAT ACA ATT AAC CCA GCT ATT  
2911 2920 2929 2938 2947 2956

A H G V D S Y I G S L E V G K K L A D I  
GCA CAT GGT GTT GAT TCT TAT ATT GGT TCA CTA GAA GTT GGT AAA TTA CCA GAT ATT  
2968 2977 2986 2995 3004 3013

V A W E P K F F G A K P Y Y V V K M G  
GTT GCT TGA GAA CCT AAA TTC TTT GGT GCA AAA OCT TAT TAT GTT GTA AAA ATG GGT  
3025 3034 3043 3052 3061 3070

V I A R C V A G D P N A S I P T C E P  
GTA ATC GCT GCT TGT GTA CCA GGT GAT CCA AAT GCT TCA ATT PCT ACA TGT GAA OCT  
3082 3091 3100 3109 3118 3127

V I M R D Q F G T Y G R L L T N T S V  
GTA ATT ATG GGT GAC CAA TTT GGA ACT TAT GGA GGT TTG TTA ACT AAT ACA TCA TTT GAT  
3139 3148 3157 3166 3175 3184

S F V S K I G L E N G I K E E Y K L E  
AGT TTT GGT TCA AAA ATT GGG TTA GAA AAT GGC ATT AAA GAG GAA TAT AAA TTA GAA  
3196 3205 3214 3223 3232 3241

K E L L P V K N C R S V N K K S M K W  
AAA GAA TTA TTA CCA GTT AAA AAT TGC CGT TCA GTA ATT AAA AAG AGT ATG AAA TGA  
3253 3262 3271 3280 3289 3298

N S A T P N L E V D F Q T F D A A V D  
AAC TCT GCG ACT CCA AAT TTA GAA GGT GAT CCG CAA ACT TTT GAT GCT GCT GAT GAT  
3310 3319 3328 3337 3346 3355

L M I  
TTA ATT ATT TAG AAA ATT GAC TAG AAT GAT CAG CTT CTG AAT TAG CTA AAA AAT TAA  
3367 3376 3385 3394 3403 3412

AAA AGA CTT CAA GTG GCA AAT ATA TAC TTG ATG AAC CTT TAA CAG AAG GGC CAT  
3424 3433 3442 3451 3460 3469

TAG CAC AAA GAT ATT TCT TAT TTT AAT TGT CAA ACT AAA ATA TTA TAT TTA TTT ATT  
3481 3490 3499 3508 3517 3526

TTC GTT ACA TTT TAA AAA AGC GGG GGA ATA ATC TTG ACT GTA TTT AAA GAA ATT TTA  
3538 3547 3556 3565 3574 3583

A P L A N I K N V E S Y Q I E N I H L  
GCT OCT CTT GCT AAC ATT AAA AAT GGT GAA AGT TAT CAA ATT GAG AAC ATT CAT TTA  
3595 3604 3613 3622 3631 3640

FIG. 3. Nucleotide and deduced amino acid sequences of the urease gene cluster of *U. urealyticum* serotype 1. Putative ribosomal binding sites are underlined. The -10 and -35 boxes of the *ureA* promoter are in italic type and underlined. The names of the corresponding polypeptides are in capital letters in bold type.

```

T S D D V L K R V I I I S S D Q N V E
ACA AGC GAC GAC GTT TTA AAA CGT GTA ATT ATC ATT TCA TCA GAT CAA AAT GTT GAA
3652 3661 3670 3679 3688 3697

Y G I R L E E D K K L M D G D I L Y K
TAT GGA ATT CGT TTA GAA GAG GAC AAA TTA ATG GAT GGT GAC ATC TTA TAC AAG
3709 3718 3727 3736 3745 3754

D D Y K L V V I R L E L S D V L I I T
GAT GAT TAT AAA CTA GTT GTT ATT AGA TTA GAG TTA TCA GAT GTA TTA ATT ATT ACA
3766 3775 3784 3793 3802 3811

A H T I G E W Q N C H N L G N R H M P
GCA CAC ACA ATT GGT GAA TGG CAA AAT TGC CAT AAT TTA GGT AAT CGT CAT ATG CCG
3823 3832 3841 3850 3859 3868

A Q F T E T Q M I V P Y D Y L V E E Y
GCT CAA TTT ACT GAA ACA CAA ATG ATC GTT CCC TAC GAC TAT TTA GTA GAA GAA TAC
3880 3889 3907 3916 3925

L Q G D N K A L Y E R K K I K L K E A F
CTT CAA GAT AAT AAA GGC CTA TAT GAA AGA AAA ATT AAA CTT AAA GAA GCA TTT
3937 3946 3955 3964 3973 3982

K H C S D A K
AAA CAC TGT AGT GAC GCT AAA TAG TGA CTA TTT AAA TTT ATT AGA CCT AAT GCA GAT C
3994 4003 4012 4021 4030 4039
ACT AAC GCA AAC TTT CCA ATC CGA ACA TTT AGT CAT TCT TTT GGA GTT GAA ACT TAT
4052 4061 4070 4079 4088 4097

ATT AGA AAA GAT ATT GTT TTT GAT GAA GAA TCA TTA ATT AAA GCG TTA CTT CTG TCA
4109 4118 4127 4136 4145 4154

TAT GAA TGA ACA ACT TAG CTT ACA ATG GTG ATT TAT TGG CAA TTT ATT ACA TTT TTA
4166 4175 4184 4193 4202 4211

AAC TAT TGC CAA AAC AAA TAA ATG CAA AAT TTT GGA AAT TGT ATC AAA TTG ATA
4223 4232 4241 4250 4259 4268

N F K G L A R E E T R E G Q R R I G Q Q
AAC TTT AAA GGT TTA GCA AGA GAG ACT CGT GAA GGT CAA CGT CGA ATT GGA CAA CAA
4280 4289 4298 4307 4316 4325

M V K I Y N E L F N C E L L V E Y A E
GAT GTA AAG ATA TAT AAT GAA CTT TTT AAT TGT GAA CTT TTA GTT GAA TAT GAT GAA
4337 4346 4355 4364 4373 4382

R I K N K K S Y G N P A V A F A L L A
AGA ATA AAA AAT AAA AAA TCT TAT GGA AAT CCT GCT GTT GCG TTT GCT TTA TTA GCT
4394 4403 4412 4421 4430 4439

M H L K A I D L K T A L Y T H L Y S T V
ATG CAT TTA AAA ATA GAT TTA AAA ACT GCT TTA TAT ACA CAT CTT TAC TCT ACA GTT
4451 4460 4469 4478 4487 4496

A A L T Q N C V R A I F L G Q V K G Q
GCT GCA TTA ACG CAA AAC TGT GTA CGT GCA ATT CCA TTA GGA CAA GTT AAG GGA CAA
4508 4517 4526 4535 4544 4553

K I I Y Q L K H V Y F D D I V N K V F
AAA ATA ATT TAT CAA CTT AAA CAT GTT TAT TTT GAT GAT ATT GPT AAT AAA GTC TTT
4565 4574 4583 4592 4601 4610

T L I L K Q I F A K I Y Q A L K L P N
ACA TTG ATT TTA AAA CAG ATT TTT GCA AAA ATA TAC CAG GCC TTG AAA TTG CCA AAT
4622 4631 4640 4649 4658 4667

G T L R H T C S I V H V I I K V L V K
GGA ACA CTA AGA CAC ACC TGT TCG ATT GTT CAT GTC ATA ATT AAA GTT TTA GTG AAA
4679 4688 4697 4706 4715 4724

H Y I N H K N T I L I F L E N L K K K
CAT TAC ATT AAT CAT AAA AAT ACC ATT TTA ATA TTT TTA GAA AAT TTA AAA AAG AAA
4736 4745 4754 4763 4772 4781

E N . UREB M K R P L I I G V G G P V G A
GAG AAT TAA TT ATG AAA AGA CCA TTA ATT GTT GTA GGT GGA OCT GTA GGA CCA
4792 4801 4810 4819 4828 4837

G K T M L I E R L T R Y L S T K G Y S
GGT AAA ACA ATG CTA ATC GAA GAA TTA ACA AGA TAC CTT TCA ACA AAA GGT TAT AGT
4849 4858 4867 4876 4885 4894

M A A I T N D I Y T K E D A R I L L N
ATG GCA GCA ATT ACT AAT GAT ATT TAC ACT AAA GAA GAT GCT AGA ATC TTA TTA AAT
4906 4915 4924 4933 4942 4951

T S V L P A D R I A G V E T G G C P H
ACT TCT GTT TTA CCA GCT GAT CGT ATT GGT GGT GAA ACA GGA GGT TCA CAT
4963 4972 4981 4990 4999 5008

T A I R E D A S M N F A A I E E M C D
ACA GCG ATT CGT GAA GAT GCT TCA ATG AAC TTT GCT GCA ATT GAA GAA ATG TGT GAT
5020 5029 5038 5047 5056 5065

K H P D L Q L F L E S G G D N L S A
AAA CAC CCT GAT TTA CAA TTA TTT TTA GAA TCT GGT GGT GAT AAT TTA TCT GCA
5077 5086 5095 5104 5113 5122

T F S P D L V D F S I Y I I D V A Q G
ACA TTT AGT CCA GAT TTA GTT GAT TTT TCA ATT TAT ATC ATT GAT GAT GCA CAA GGA
5134 5143 5152 5161 5170 5179

E K I P R K G G Q G M I K S D L F I I
GAA AAA ATT CCT CGT AAG GGT GGT CAA GGG ATG ATT AAA TCA GAT GAT TTA TTT ATT
5191 5200 5209 5218 5227 5236

N K V D L A P Y V G A N V E V M K A D
AAT AAA GTT GAT TTA GCT CCT TAT GTT GGT GCT AAT GTA GAA GTA ATG AAA GGC GAT
5248 5257 5266 5275 5284 5293

T L K S R G N K D F F V T N L K T D E
ACA TTA AAA TCA CGT GGT AAT AAA GAT TTC TTT GTA ACG AAT TTA AAA ACA GAT AAA
5305 5314 5323 5332 5341 5350

G L K S V A D W I E K R L Q L A L L E
GCT TTA AAA TCT GTT GCT GAT TGA ATT GAA AAG CGT TTG CAA TTA GCT TTA CTT GAA
5362 5371 5380 5389 5407

E . URED M I L N K E K I K N Y A A Y
GAA TAA AGT TAA CAA ATG ATT TTA AAT AAA GAA AAA ATT AAA AAT TAT GCT GCT TAT
5419 5428 5437 5446 5455 5464

L Y I K V A Y D Q A H S K M A H T V Y
TTA TAC ATT AAA GTA GCA TAT GAT CAA GCT CAT TCA AAA ATG GCA CAT ACT GTC TAT
5476 5485 5494 5503 5512 5521

F T N F Y R S S K P L F L D E E D P I
TTT ACG AAT TTT TAT CGT TCA TCT AAA OCT TTA TTT TTA GAT GAA GAA GAT CCA ATC
5533 5542 5551 5560 5569 5578

N P C F Q T I S M G G G Y V S G E V Y
AAC OCT TGT TTT CAA ACA ATT AGT ATG GGT GGT GCA TAT GTT TCA GGT GAA GTT TAT
5590 5599 5608 5617 5626 5635

R S D F E I N D A A C C I I T T Q S S
CGT TCT GAT TTT GAA ATT AAT GAT GCT GCT TGT TGT ATC ATT ACA ACA CAA TCC TCA
5647 5656 5665 5674 5683 5692

R K A
CGT AAA GCT
5704

```

FIG. 3—Continued.

**Identification of a promoter region for *ureA*.** The positioning of the genes *ureA*, *ureB*, and *ureC* immediately adjacent to each other and the spacing between ORF1 and *ureA* suggest that the three genes for structural subunits may be under the control of a single promoter upstream of *ureA*. The transcription initiation sites of *ureA* of *U. urealyticum* serotypes 1 and 8 were determined by primer extension from purified RNAs (Fig. 5). The extremely low yield of RNA purification required a large volume of ureaplasma culture (10 liters). Primers were chosen close to the 5' ends of the urease genes within *ureA*. The transcription initiation site was determined by comparison of the size of the cDNA synthesized by primer extension with the sizes of DNA fragments generated by sequencing reactions performed with the pS1 or pC6b plasmid. The +1 nucleotide (C) preceding the ATG start codon is underlined in the following sequence: 5'-TAAAATTGCATTATTAC-3' for both serotypes. The nucleotide sequences of the -10 and -35 regions are TAAATA and TGTATT for serotype 1 and TAA AAT and TGCAAC for serotype 8, respectively. A Shine-Dalgarno-type sequence was found, in both cases, upstream of the initiation codon (Fig. 3).

**Expression of urease genes in the PR101 opal suppressor strain of *E. coli*.** The presence of UGA codons that encode Trp in *U. urealyticum* did not allow urease expression from the

plasmid pC6b in a standard (nonsuppressor) strain of *E. coli* (3). In order to determine if the pS1 and pC6b plasmids contained all the genetic information required for urease expression, the PR101 opal suppressor strain of *E. coli* was electrotransformed with each of these two plasmids and pC61. The plasmid pC61, containing only truncated genes for structural subunits (4), was used as a negative control. The selected transformants were inoculated in M9 medium containing 10  $\mu$ M NiCl<sub>2</sub> (which is necessary for the urease nickel metallo-center biosynthesis) and IPTG (which is required for induction of the suppressor system). Urease activity of the transformants was assessed by using the indole-urea indicator medium. This activity, as evidenced by a shift from yellow to red of the pH indicator in the medium, was detected only in tubes containing *E. coli* that had been transformed with pC6b and not in tubes containing *E. coli* transformed with pS1 or pC61. This shift usually appeared within 1 to 3 h of incubation. In addition, the level of urease activity was quantitated by the Berthelot reaction. When cells containing pC6b were cultivated in M9 medium without NH<sub>4</sub>Cl and supplemented with urea at 1 or 10 mM, the urease activity was 1.8  $\pm$  0.5 and 4.3  $\pm$  0.8  $\mu$ mol of NH<sub>3</sub> per min per mg of protein, respectively. In M9 medium with NH<sub>4</sub>Cl and supplemented with 10 mM urea, urease ac-

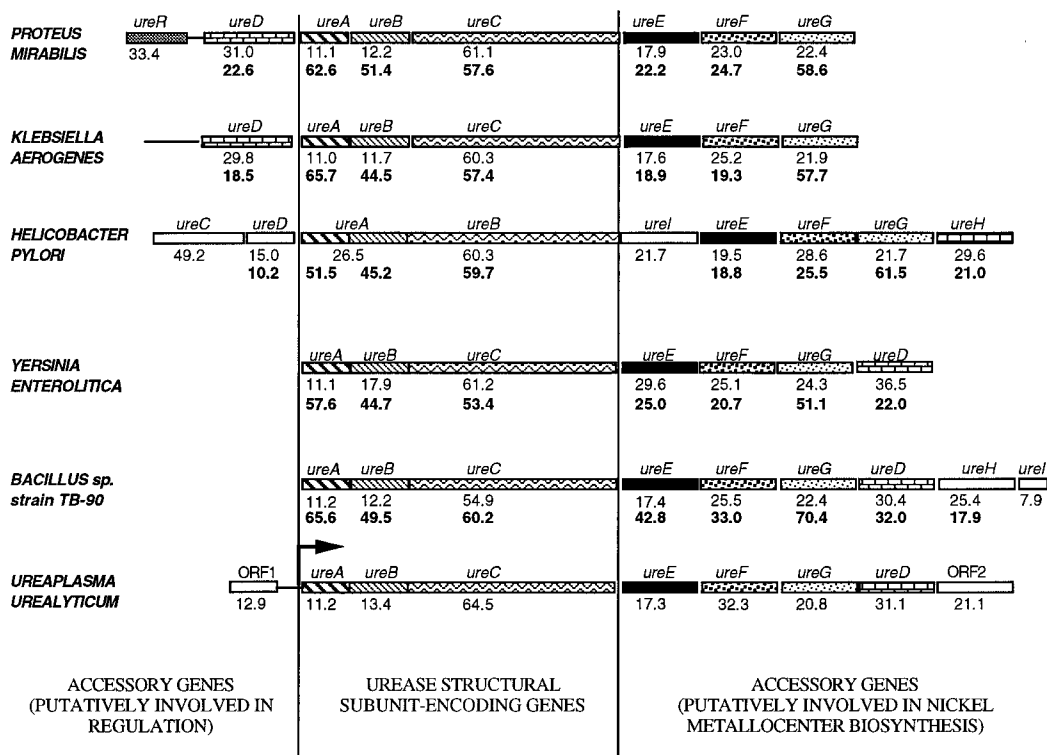


FIG. 4. Comparison of the urease gene cluster from *P. mirabilis*, *K. aerogenes*, *H. pylori*, *Y. enterocolitica*, *Bacillus* sp. strain TB-90, and *U. urealyticum*. The numbers under the genes indicate the molecular mass (in kilodaltons) of the corresponding deduced polypeptide. The percentages of identity between these polypeptides from *P. mirabilis*, *K. aerogenes*, *Y. enterocolitica*, *Bacillus* sp. strain TB-90, and *H. pylori* with those from *U. urealyticum* are indicated in bold type. The ORF1 product from *U. urealyticum* was compared with the products of the *ureD* genes from *P. mirabilis* and *K. aerogenes* and the *ureH* gene from *H. pylori*. The UreA polypeptide from *U. urealyticum* is compared with the N-terminal end of the UreA polypeptide from *H. pylori*; the UreB polypeptide from *U. urealyticum* is compared with the C-terminal end of the UreA polypeptide from *H. pylori*. The *U. urealyticum* ORF2 product was compared with the *ureH* product from *Bacillus* sp. strain TB-90. The arrow indicates the *ureA* promoter of *U. urealyticum*. The sequence data for *U. urealyticum* were obtained from pS1 (from ORF1 to *ureG*) and from pC6b (for *ureD* and ORF2).

tivity was slightly higher with a value of  $7.1 \pm 1.8 \mu\text{mol}$  of  $\text{NH}_3$  per min per mg of protein.

The lack of expression with pS1 could be tentatively explained by the truncation of *ureD* at its 3' end. The requirement of genes at the 3' end of the urease gene cluster was further investigated by nested deletion mutagenesis of pC6b.

**Contribution of *ureD* expression to catalytically active urease in *E. coli*.** Nested deleted mutations of pC6b downstream from *ureG* were obtained by using exonuclease III. The mutant plasmids were selected upon analysis of their size on agarose gels after electrophoresis and were introduced into *E. coli* PR101 by electroporation. Their ability to allow urease expres-

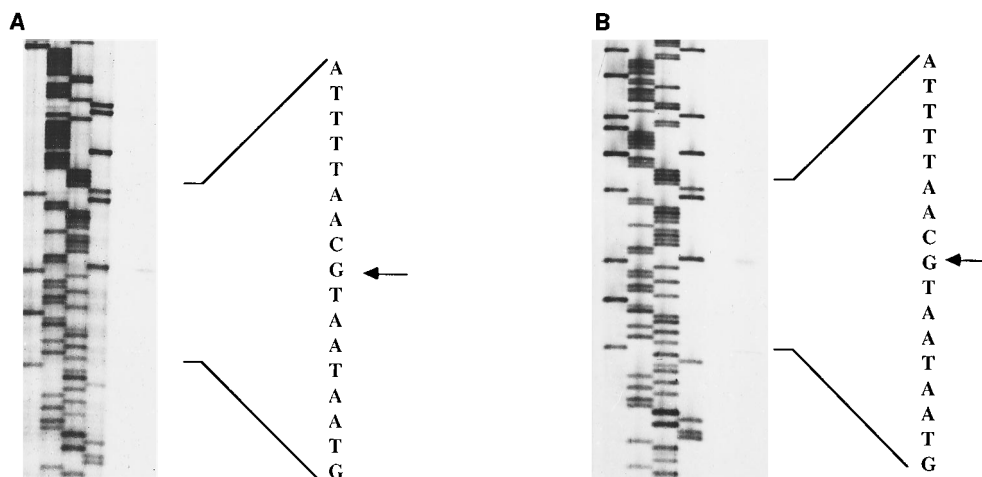


FIG. 5. Determination of the transcription initiation site of urease genes from *U. urealyticum* serotype 1 (A) and serotype 8 (B). The transcription initiation site for the *ureA* gene was determined by primer extension from total purified RNAs. Sequencing was performed in parallel with the same primers as for the extension from RNAs using pS1 (A) or pC6b (B) as the DNA template. The arrows indicate the transcription initiation site.

TCA GAT TTA TTC ATC ATC AAT AAA GIT GAT TTA GCT CCT TAT GIT GGT GCT  
 10 19 28 37 46  
 AAT GTG NAA GTA ATG AAA GCT GAT ACA TTA AAA TCA CGT GGT AAT AAA GAT TTC TTT  
 61 70 79 88 97 106  
 GTA ACA AAT TTA AAA ACA GAT GAA GGT CTA AAA TCT GGT TGA GTT GAA AAA  
 118 127 136 145 154 163  
**UreH** M I L S K E  
 CGT TTA CAA TTA GCT TTA CTT GAA GAA TAA GAC TAA CAA ATG ATT TTA AGT AAC GAA  
 175 184 193 202 211 220  
 K I N N Y A A Y L Y I K V A Y D E A H  
 AAA ATT AAC AAT TAT GCT GCT TAT TTA TAC ATT AAA GTA GCA TAT GAT GAA GCA CAC  
 232 241 250 259 268 277  
 N K M A H T V Y F T N F Y R S S K P L  
 AAC AAA ATG GCG CAT ACT GTG TAT TTC ACT AAT TTC TAT CGT TCA TCA AAA CCA CTA  
 289 298 307 316 325 334  
 F L D E E D P I N P C F Q T I S M G G  
 TTT TTA GAT GAA GAA GAC CCA ATT AAC COC TGT TTT CAA ACT ATT AGT ATG GCG GGG  
 346 355 364 373 382 391  
 G Y V S G E V Y R S D F E V E A N A R  
 GGT TAT GTA TCT GGT GAA GTG TAT CQC TCT GAT TTT GAA GTT GAA GCA AAT GCA CGT  
 403 412 421 430 439 448  
 C I I T T Q S S A K A Y K A V D G K T  
 TGC ATT ATT ACT ACG CAA TCA TCA GGC AAA GCT TAT AAA GCA GAT GGT AAC AAA ACT  
 460 469 478 487 496 505  
 S E Q H T N I T L G K N S I L E Y I S  
 TCA GAA CAA CAC ACA AAT ATT ACA TTA GGA AAA AAT AGT ATT TTA GAA TAC ATA AGT  
 517 526 535 544 553 562  
 D N V I V Y E D G K F A Q F N N F K M  
 GAT AAT GTA ATT GTG TAT GAA GAT GCA AAA TTT GGC CAA TTT AAC AAT TTT AAA ATG  
 574 583 592 601 610 619  
 D S T A T L I Y T E C F G P G W S P H  
 GAT TCA ACT GCT ACA CTA ATT TAC ACA GAA TGT TTT GGT CCT GGT TGA TCG CCA CAT  
 631 640 649 658 667 676  
 G S A Y Q Y E K M Y L N T K I Y Y D N  
 GGA TCT GCT TAT CAA TAC GAA AAA ATG TAT TTA AAT ACT AAA ATA TAT TAT GAC AAT  
 688 697 706 715 724 733  
 K L V L F D N L K F Q P R K N D E S A  
 AAA TTG GTT TTA TTT GAT AAT TTA AAA TTT CAA CGT AAA AAT GAT GAA TCA GCA  
 745 754 763 772 781 790  
**Δ**  
 F G I M D G Y H Y C G T M I V I N Q E  
 TTT GGT ATT ATG GAT GGT TAT CAC TAT TGT GGA ACA ATG ATT GTA ATT AAC CAA GAA  
 802 811 820 829 838 847  
 V V E E D V I K I R D L V K E K Y P D  
 GGT GGT GAA GAA GAT GTG ATT AAA ATT CGT GAT TTA GTT AAG GAA AAA TAT CCC GAT  
 859 868 877 886 895 904  
 M D M I F G V S R M D I P G L G L R V  
 ATG GAT ATG ATA TTT GGG GTA TCA GCA ATG GAT ATT CCT GGA TTA GGA TTA CCA GTT  
 916 925 934 943 952 961  
 L A N T Y Y H V E K I N A V A H D Y F  
 TTA GGC AAT ACT TAT TAC CAT GTT GAA AAA ATT AAT GCT GTT TCA CAT GAT TAC TTT  
 973 982 991 1000 1009 1018  
 R R K L F N K K P L I L R K P  
 AGA AGA AAA TTA TTC AAT AAA AAA CCA TTA ATT TTA CGA AAA CCA TAG AAG AT TTA  
 1029 1038 1047 1056 1065 1074  
 AAA ACC TTA AAA ACG TAC TTG TTT TTA AGG TTT TTT GTT ACT AAA AAA TTC TTA ATA  
 1086 1095 1104 1113 1122 1131  
**UreJ** M N L N H K E D D S  
 AAT TTA TAA AAT ATT TAT ATA ATA TAT ATG AAT TTA AAC CAC AAG GAG GAT GAT TCC  
 1143 1152 1161 1170 1179 1188  
 M A N S Q K V I D V S N A H Y N L N L  
 ATG GCT AAC TCT CAA AAA GTA ATT GAT GTT TCA AAT GCA CAT TAT AAC TTA AAT TTA  
 1200 1209 1218 1227 1236 1245  
 E L G S V Y A Q Y A H I A D D Q F S M  
 GAA TTA GGA AGT GTG TAT GCT CAA TAT GCT CAT ATA GCT GAT GAT CAA TTT AGT ATG  
 1257 1266 1275 1284 1293 1302  
 P F L A K F I N D L S N D K L G V H K  
 CCT TTT TTA GCA AAA TTT ATT AAT GAT TTA AGT AAT GAT AAA TTA GGT GGT CAC AAA  
 1314 1323 1332 1341 1350 1359  
**Δ**  
 D L I S E Y A R K I E I P L H T K F S  
 GAT TTA ATT TCA GAA TAT GCA CGT AAA ATT GAA ATT CCA TTA CAT ACT AAA TTT AGT  
 1371 1380 1389 1398 1407 1416  
 V D V S F K P T D P K E L V K H I L E  
 GTA GAT GTT AGT TTT AAA CCT ACA GAT CCT AAA GAA TTA GTA AAA CAC ATC TTA GAA  
 1428 1437 1446 1455 1464 1473  
 T E Q K V R K H V A N M A K V C L E E  
 ACA GAA CAA AAA GTT CGT AAA CAC GAT GCT AAT ATG GCT AAG GTA TGC TTA GAA GAA  
 1485 1494 1503 1512 1521 1530  
 G D F E T F S F V K W F V D D G I K D  
 GGT GAC TTT GAA ACT TTT AGT TTC GTT AAA TGA TTT GTA GAT GAT GGT ATT AAA GAT  
 1542 1551 1560 1569 1578 1587  
 F D D V R T I H D F F E N G N N L Q  
 TTT GAT GAT GTT CGC ACA ATT CAT GAT TTC TTT GAA AAT GGC AAT AAT AAT TTA CAA  
 1599 1608 1617 1626 1635 1644  
 V E Y A I R Q I F K A N E A W G R K I  
 GGT GAA TAC GCT ATT COC CAA ATA TTT AAA GCA AAT GAA GCT TGA GGA AGA AAA ATA  
 1656 1665 1674 1683 1692 1701  
 I  
 ATT TAG CTA TTT ATC TTA TAT ATT AGA TAA TAA AAA ACT ATT TGT AAG GTC ATA AAA  
 1713 1722 1731 1740 1749 1758  
 AGA CCC ATA CAA GTG GTT TTT TAT ATT GTG TTT AAG ATG TAA AAG TTT AAT TTT TTA  
 1770 1779 1788 1797 1806 1815  
 GTG AAA AAT AAT GTT TTT TTG CTA CAA TGT ATA CTC GCA AAT TTT AAA AAT TTA ATT  
 1827 1836 1845 1854 1863 1872  
 GTT GTT ATA AAA TTA TTT TTT ATT TTA TTT GCT AAT ACA TAT CAC ATC TAA AAT ATA  
 1884 1893 1902 1911 1920 1929  
 AAT AAT TTT TAC ACA ATT AGT ATA TTT AGG AAA TCC TCG TGA ATG AAA TTA TCG AAA  
 1941 1950 1959 1968 1977 1986  
 AAC AAA AAA TTT TTT AAC AAT GTC ATT AAG TGG AGT TTT ATT AAC TAC TAG CGT GGT  
 1998 2007 2016 2025 2034 2043  
 TGC GAT TGC ATC ATC TTA TGC TAA AAA ACA AAC AAA AAT TGA AAG TGT TAG GCA TAG GC  
 2055 2064 2073 2082 2091 2100

FIG. 6. Sequence of the region downstream of *ureG* from the urease gene cluster of *U. urealyticum* serotype 8. The nucleotide sequence of the 3' end of the insert from plasmid pC6b containing the urease gene cluster of serotype 8 from *U. urealyticum* was determined. The names of the deduced polypeptides are in bold type. The  $\Delta$  symbols indicate the positions of the 3' end of the nested deletions obtained from pC6b and described in the text.

sion was assessed in indole-urea indicator medium. Transformants containing plasmid pC6b(1) $\Delta$ 1 were urease negative, whereas the transformants containing plasmid pC6b(2) $\Delta$ 1 were urease positive. The DNA insert in pC6b(1) $\Delta$ 1 and pC6b(2) $\Delta$ 1 was found to be 5.2 and 5.8 kbp long, respectively. In order to localize the region of inactivation, the 3' ends of the inserts of these two plasmids were sequenced. The pC6b(2) $\Delta$ 1 plasmid, which is urease positive, contains a complete *ureD* gene (nucleotides 205 to 1068 on Fig. 6) followed by a truncated ORF named ORF2 (Fig. 1). The pC6b(1) $\Delta$ 1 plasmid, which is urease negative, contains a 3'-truncated *ureD* gene (Fig. 1). The positions of deletions are precisely indicated with the symbol  $\Delta$  in Fig. 6. This result demonstrates that *ureD* is essential for urease activity, whereas ORF2 is not. Additional sequencing on pC6b revealed the complete sequence of ORF2 (nucleotides 1161 to 1709 on Fig. 6). At the 3' end of this ORF,

no other obvious ORF could be found within 396 additional nucleotides (Fig. 6).

DISCUSSION

The detection of urease genes from different strains of *U. urealyticum* showed the presence of two distinct *Hind*III profiles, in agreement with the clustering of strains into two biotypes. Sequence analysis of the urease gene cluster from serotype 1 indicated that this polymorphism is due to the lack of a *Hind*III site within *ureC* for strains of biotype 1. This genetic variation between the two biotypes also exists within the nucleotide sequence upstream of *ureA* and, a posteriori, explains the specificity for the strains of biotype 2 of the PCR assay that was described previously with one of the primers within this region (3). In addition, this genetic variation, in particular

within the promoter region of *ureA*, may correspond to different levels of urease expression between the strains of the two biotypes, as suggested by Robertson and Chen (36) on the basis of the different sensitivities to manganese; this remains to be determined.

Comparison of urease genes from serotypes 1 and 8 showed minor differences. In particular, the deduced UreB and UreC polypeptides from serotype 1 have a lower molecular weight than those of serotype 8, this finding concurring with the difference previously observed by gel electrophoresis of the ureases from these serotypes (18).

Analysis of the organization of the *U. urealyticum* urease gene cluster revealed similarities to that found in other ureolytic bacteria. Indeed, the succession of genes from *ureA* to *ureG* has been found in all the urease gene clusters (Fig. 4). In particular, there are three genes encoding structural subunits, *ureA*, *ureB*, and *ureC*, as was found for *E. coli* (17), *P. mirabilis* (23), *Klebsiella pneumoniae* (12), and *K. aerogenes* (30); for *H. pylori*, the *ureA* and *ureB* genes are fused in a single gene, *ureA*. Furthermore, downstream of *ureC*, three genes coding for accessory proteins (*ureE*, *ureF*, and *ureG*) were found similar to those in other ureolytic bacteria (for reviews, see references 11 and 29). The COOH extremity of the UreE polypeptide does not include a polyhistidine tail that could bind nickel, as was shown for *K. aerogenes* and *P. mirabilis* (26, 30, 41); however, this region is also highly alkaline being composed essentially of lysine residues (137-KALYERKKIKLKEAFKHCSDAK-166 [alkaline amino acid residues are shown in boldface type]). An ATP- or GTP-binding motif was found at the amino-terminal end of UreG (G-16PVGAGKT-23) containing a glycine-rich region which could result in the formation of a flexible loop between a beta-strand and an alpha-helix. This result concurs with the finding that this polypeptide is required in the energy-dependent biosynthesis of the metallocenter, as was established for *K. aerogenes* (25). In addition, the high degree of conservation of UreG between the different ureolytic bacteria is quite striking and suggests a strong selection pressure on the corresponding gene.

The other features of the *U. urealyticum* urease gene cluster are less conserved among ureolytic bacteria. *U. urealyticum* *ureD* was named on the basis of the significant homology (32% identity [Fig. 4]) found with the UreD polypeptide from *Bacillus* sp. strain TB-90. Using nested deletion experiments, we have shown that *ureD* is necessary for urease activity. The products of *ureH* in *H. pylori* and of *ureD* in *Yersinia enterocolitica* have a limited degree of homology (approximately 20% of identity) with the UreD polypeptide from *K. aerogenes* (11, 14). On the basis of this finding, it has been proposed that these polypeptides may be homologs. A recent publication has established precisely the function of UreD in *K. aerogenes* (32): this polypeptide is proposed to control the activating steps by preventing premature Ni binding to the apoenzyme that lacks CO<sub>2</sub>, a conformation expected to be found in low CO<sub>2</sub> concentration.

In *U. urealyticum*, another ORF (ORF2) was found downstream from *ureD*, and significant homology with the deduced polypeptide could not be found in databases or with UreH and UreI of *Bacillus* sp. strain TB-90. Because of its homology with other nickel transport proteins, including *H. pylori* NixA, *Bacillus* UreH polypeptide has also been proposed to be a Ni transporter (28, 46). It is possible that the gene encoding the putative Ni transporter in *U. urealyticum* is not directly linked to the urease gene cluster, which would be similar to the organization recently described for *H. pylori* (28). It will therefore be of interest to test whether complementation of *E. coli*

PR101 containing pC6b with a gene homolog to *nixA* might induce an increased urease activity.

While we have demonstrated that ORF1 and ORF2 are not necessary for urease activity in *E. coli*, we cannot exclude the possibility that these ORFs belong to the *U. urealyticum* urease gene cluster and that *E. coli* polypeptides have the capacity to complement their function in this heterologous system.

Although there is now extensive information concerning urease gene cluster organization, there is little data about the localization of the promoters involved in the expression of these genes (12, 16, 17). We have localized the promoter region controlling the expression of *ureA* and possibly at least two other urease genes (*ureB* and *ureC*) of serotypes 1 and 8 from *U. urealyticum*. For both *ureB* and *ureC*, a Shine-Dalgarno-type sequence was detected upstream of the initiation codon. The expression levels of urease genes in *U. urealyticum* therefore differs from those described for the plasmid-encoded urease locus of the members of the family *Enterobacteriaceae*, in which the promoter for the structural genes is located upstream of *ureD* (17). Although no obvious terminator could be found within the urease gene cluster, it is possible that *U. urealyticum* accessory genes are not cotranscribed with structural genes, which is suggested in particular by the intergene spacing between *ureC* and *ureE*. A nitrogen regulatory system (NTR) has been shown to be involved in the regulation of expression of the *K. aerogenes* and *K. pneumoniae* ureases (2, 12). Interestingly enough, a sequence (5'-A-4182TGGTGATT TATGGCA-4198-3') highly homologous to the consensus sequence (5'-CTGGYAYRNNNTTGA-3') (1, 21) of promoter regions controlled by the NTR system was found upstream of *ureF*. This result suggests that *ureF* expression at least could be under the control of an NTR-like system; this hypothesis remains to be verified.

The lack of a genetic system for gene transfer in ureaplasmas combined with the lack of a defined medium for growth is hampering the effort to directly measure urease gene expression in *U. urealyticum*. For that reason, we have performed a set of experiments in the suppressor strain PR101 of *E. coli*. This report is the first time that an ureaplasma antigen was expressed in *E. coli*. The suppressor system that was used allowed us to circumvent the problem of tryptophan being encoded by TGA in mycoplasmas (for a review, see reference 6). In addition, by performing primer extension experiments, we verified that the promoter of structural genes on pC6b is functional in *E. coli* and is driving the expression of at least *ureA* (data not shown). The obtained urease expression indicates that suppression is effective, although there are 12 TGA triplets encoding Trp from *ureA* to *ureD*, which might result in the synthesis of truncated products and in problems of stoichiometry between urease subunits. Under the same conditions of culture (M9 medium supplemented with 10 μM NiCl<sub>2</sub>), the level of urease expression that was obtained in the suppressor strain is similar to that reported for the *H. pylori* urease gene cluster expressed in *E. coli* (28) and corresponds to approximately a third of that obtained with the *Bacillus* urease genes expressed in *E. coli* (27). Considering the necessity of efficient suppression, the urease activity that we report is relatively high and might also be related to previous findings that had established that the specific activity of the ureaplasma urease in vivo was much greater than those of other bacterial ureases (5, 33, 42).

Finally, we have obtained results describing the organization of urease genes in *U. urealyticum* and the expression of these genes in a suppressor strain of *E. coli*. Since urease activity is easily quantified, these genes might allow us to compare the



efficiencies of different suppressor systems for ultimately selecting hosts for mycoplasmal gene expression.

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