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

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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_1F_0 ATPase (40). In addition to this key role in ureaplasmal 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 ureaplasmal urease may be a factor of virulence, as has been demonstrated for the urease of *Proteus mirabilis* (22).

Three structural subunits of the ureaplasmal purified enzyme were characterized as 72-kDa (UreC), 14-kDa (UreB), and 11-kDa (UreA) polypeptides (44). The native form of the ureaplasmal 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 *Eco*RI 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

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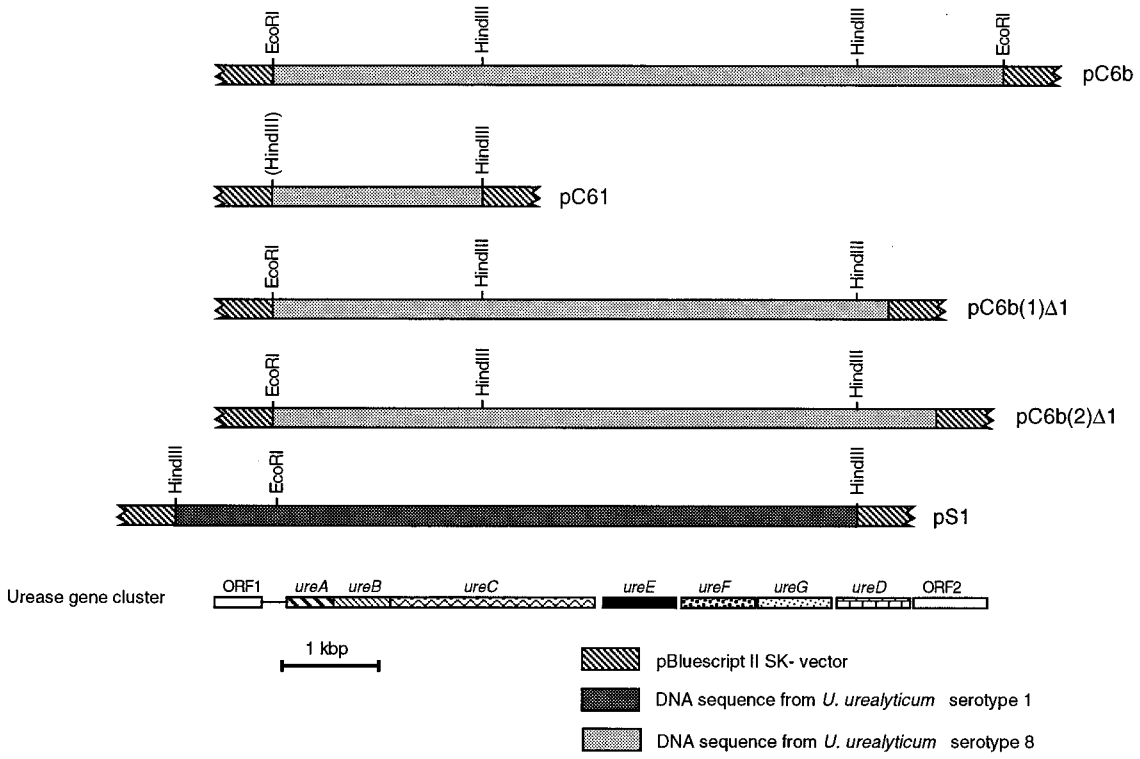

FIG. 1. Schematic representation of plasmids used in this study. The pC6b and pC61 plasmids were previously described (3, 4). The other plasmids were obtained during this study. The organization of the urease gene cluster is indicated, showing the genetic content of each plasmid.

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_2PO_4 [0.25 g/liter], K_2HPO_4 [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*$^+$ *lacI*^{q} *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 *Eco*RI site of pBluescript II SK− (Fig. 1). To construct pC61, a 2.4-kbp *Hin*dIII DNA fragment (IC61), containing *ureA*, *ureB*, and truncated *ureC* genes (4), was subcloned at the *Hin*dIII 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-^{32}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 electrophoresis, 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 *Sac*I and *Not*I or *Sac*I and *Sma*I. 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 AACGTCTTCTTGCAACGTCA-3′ for serotype 1 (which corresponds to the reverse complementary sequence of nucleotides 982 to 1006 [see Fig. 3]) and 5′-ACCTCTAGCTAAACGTCTTCTTGC-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 $NiCl_2$ 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

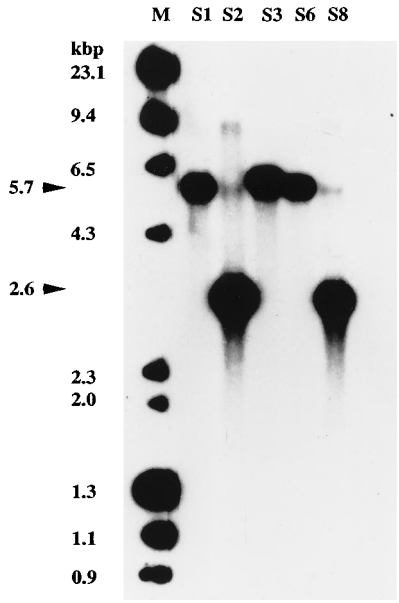

FIG. 2. Restriction fragment length polymorphism among the *Hin*dIII profiles of urease genes from the two *U. urealyticum* biotypes. Genomic DNAs from *U. urealyticum* biotype 1 (serotype 1 [lane S1], serotype 3 [lane S3], and serotype 6 [lane S6]) and biotype 2 (serotype 2 [lane S2] and serotype 8 [lane S8]) were digested with *Hin*dIII 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 *Hin*dIII and ϕX174 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 NH_4Cl). Urease activity was expressed as micromoles of 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 *Hin*dIII. 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 *Hin*dIII, 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−. Ligation products were transformed into *E. coli* DH5α. 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 *Hin*dIII site was found within *ureC*, in contrast to the *Hin*dIII 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 enterolitica* (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.

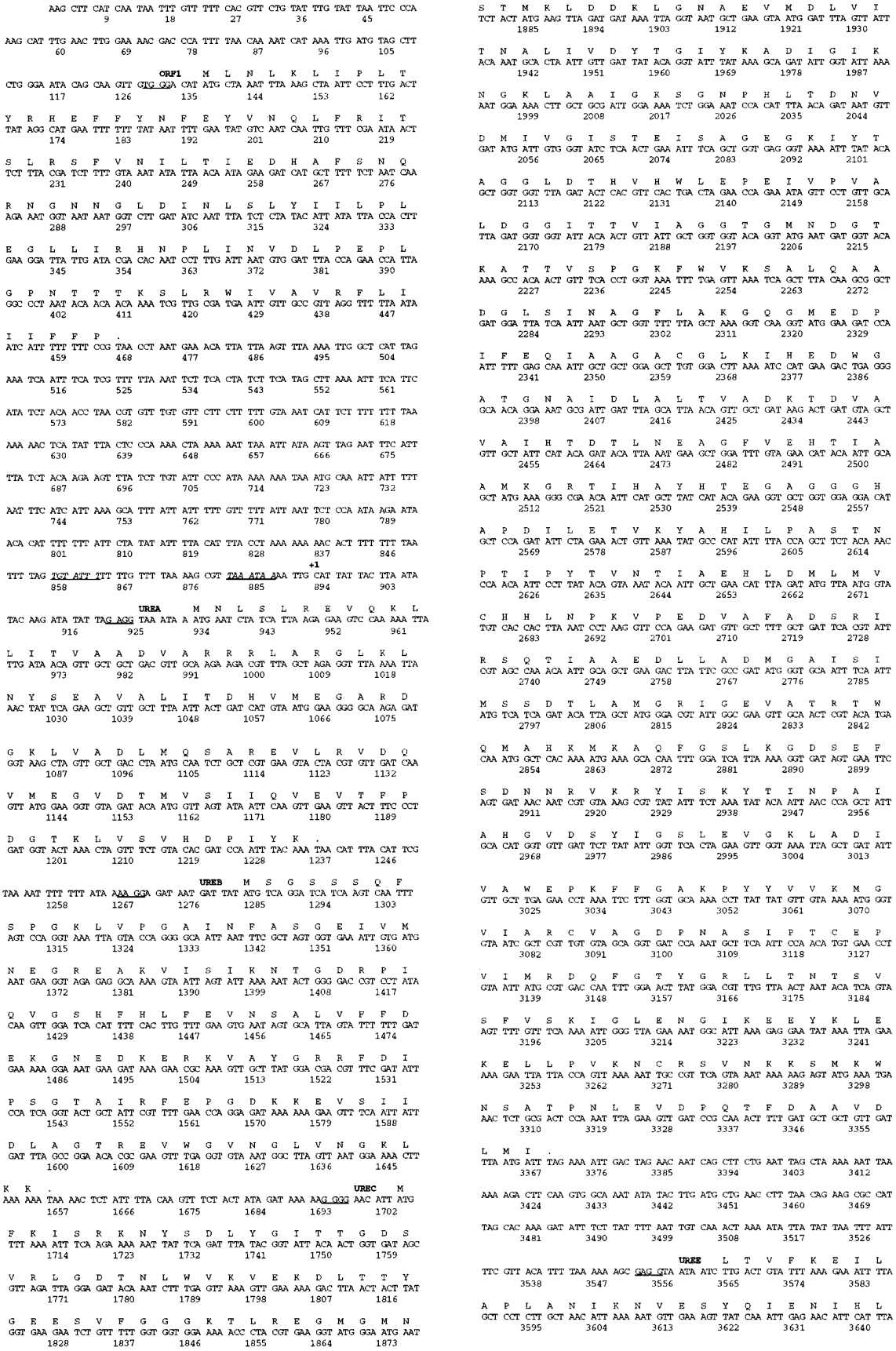

FIG. 3. Nucleotide and deduced amino acid sequences of the urease gene cluster of *U. urealyticum* serotype 1. Putative ribosomal binding sites are underlined. The −10 and −35 boxes of the *ureA* promoter are in italic type and underlined. The names of the corresponding polypeptides are in capital letters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

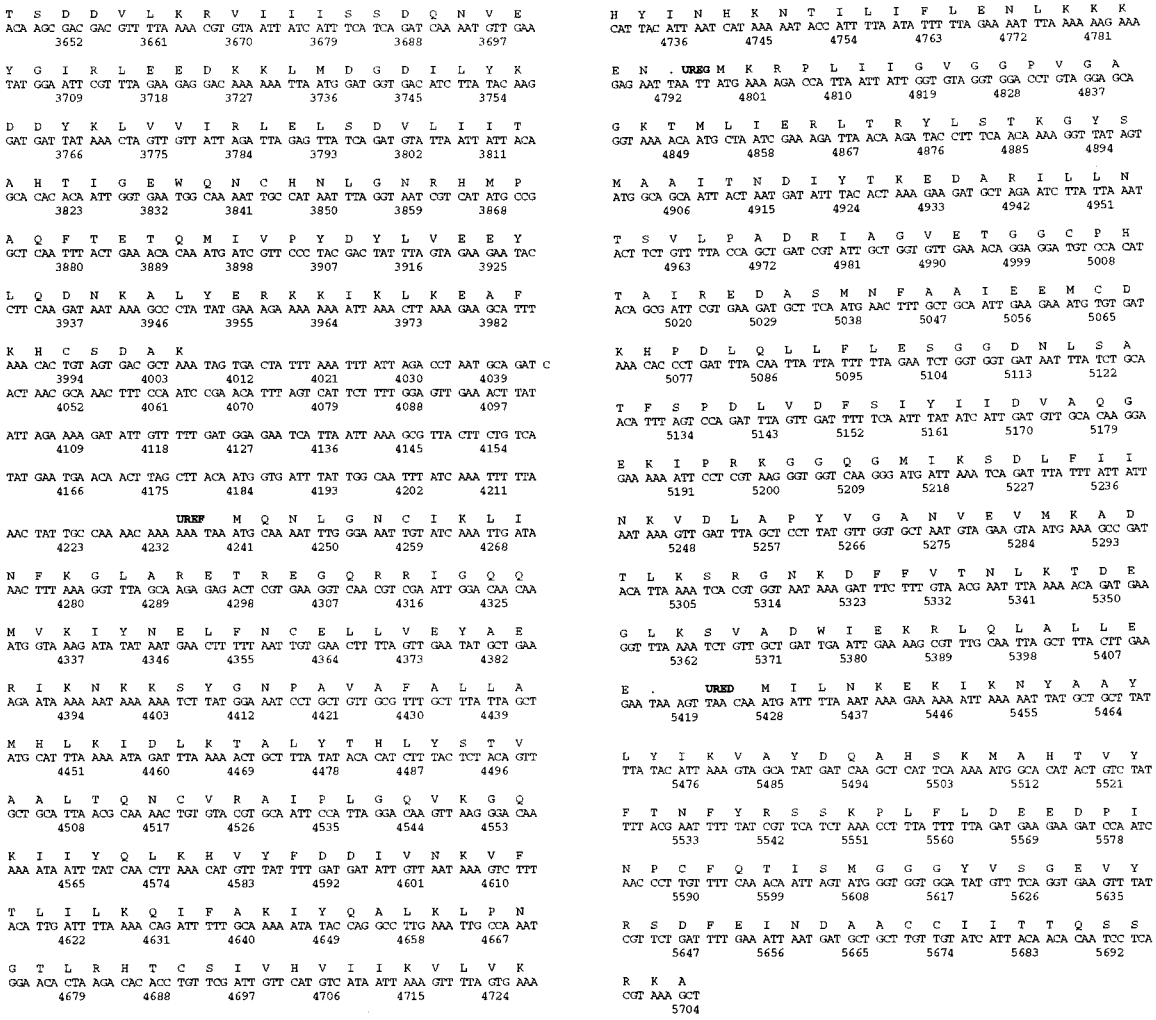

FIG. 3—*Continued.*

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 ureaplasmal 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 μM $NiCl_2$ (which is necessary for the urease nickel metallocenter 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_4Cl and supplemented with urea at 1 or 10 mM, the urease activity was 1.8 ± 0.5 and 4.3 ± 0.8 μmol of NH_3 per min per mg of protein, respectively. In M9 medium with NH_4Cl and supplemented with 10 mM urea, urease ac-

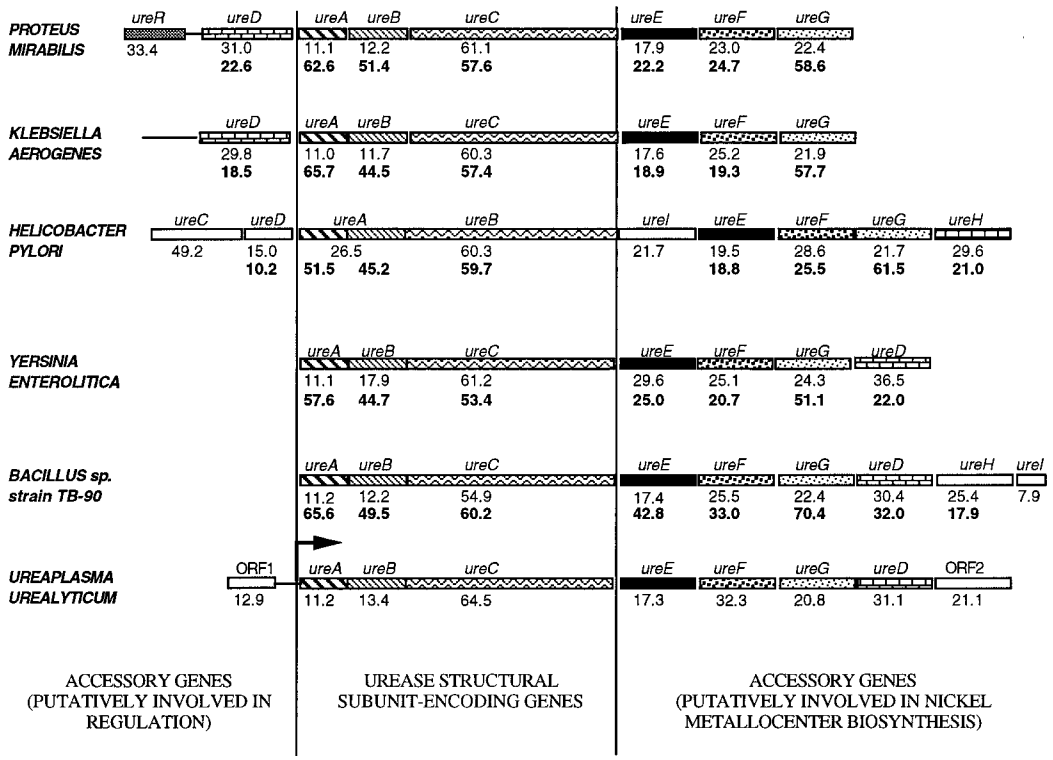

FIG. 4. Comparison of the urease gene cluster from *P. mirabilis*, *K. aerogenes*, *H. pylori*, *Y. enterolitica*, *Bacillus* sp. strain TB-90, and *U. urealyticum*. The numbers under the genes indicate the molecular mass (in kilodaltons) of the corresponding deduced polypeptide. The percentages of identity between these polypeptides from *P. mirabilis*, *K. aerogenes*, *Y. enterolitica*, *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 ± 1.8 μmol of 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-

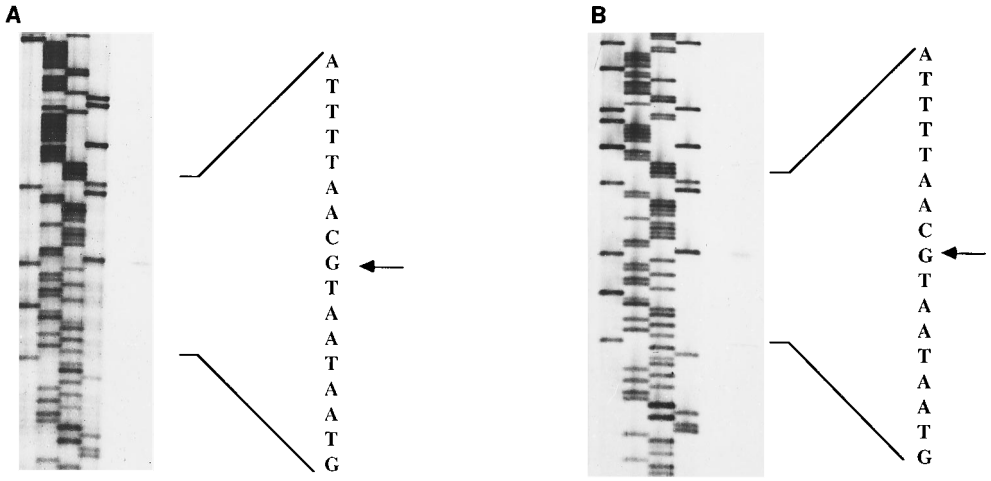

FIG. 5. Determination of the transcription initiation site of urease genes from *U. urealyticum* serotype 1 (A) and serotype 8 (B). The transcription initiation site for the *ureA* gene was determined by primer extension from total purified RNAs. Sequencing was performed in parallel with the same primers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

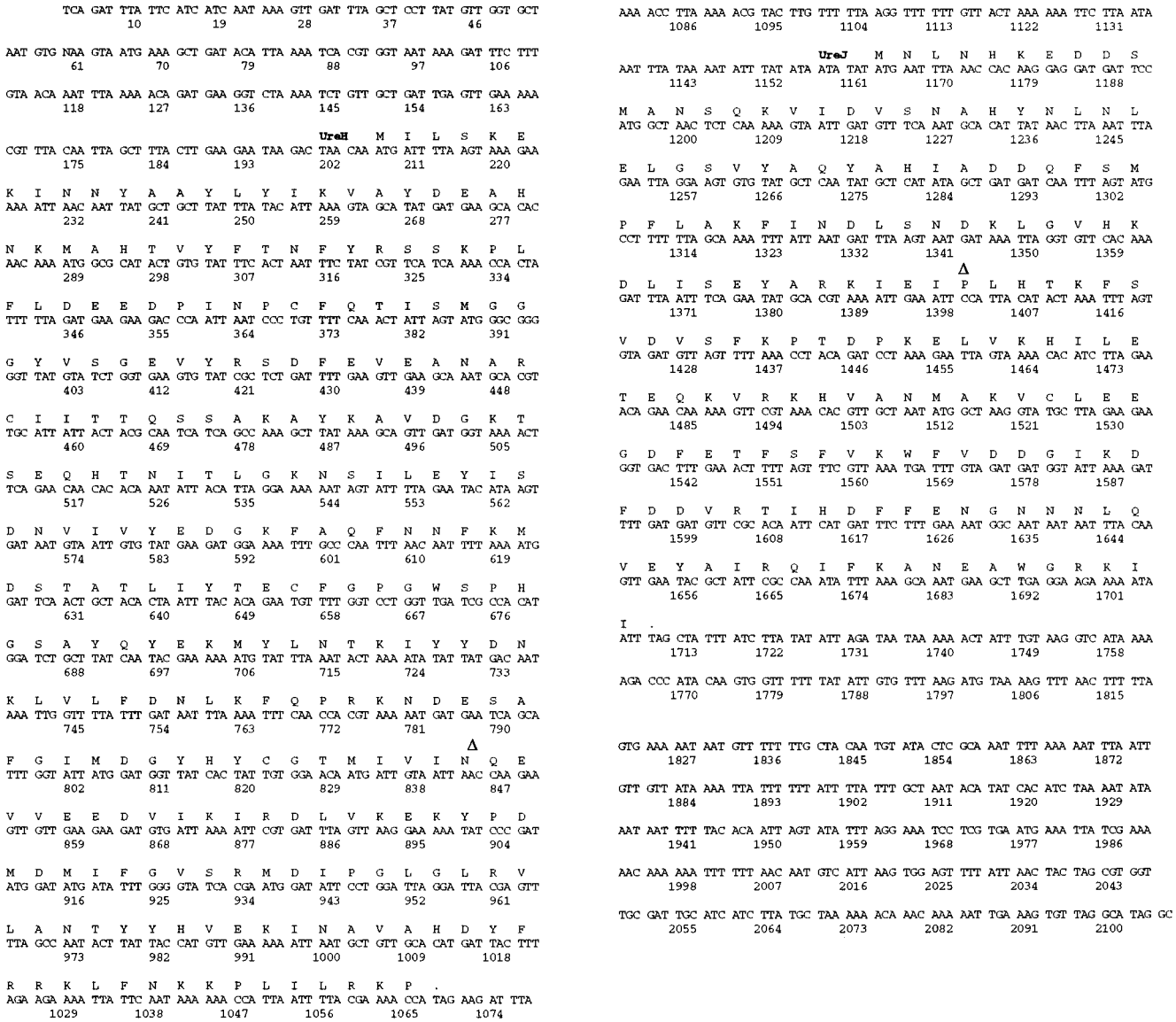

FIG. 6. Sequence of the region downstream of *ureG* from the urease gene cluster of *U. urealyticum* serotype 8. The nucleotide sequence of the 3′ end of the insert from plasmid pC6b containing the urease gene cluster of serotype 8 from *U. urealyticum* was determined. The names of the deduced polypeptides are in bold type. The Δ 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)Δ1 were urease negative, whereas the transformants containing plasmid pC6b(2)Δ1 were urease positive. The DNA insert in pC6b(1)Δ1 and pC6b(2)Δ1 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)Δ1 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)Δ1 plasmid, which is urease negative, contains a 3′-truncated *ureD* gene (Fig. 1). The positions of deletions are precisely indicated with the symbol Δ 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 *Hin*dIII 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 *Hin*dIII 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-**K**ALYE**RKKIKLK**EAF**KH**CSDA**K**-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**-16PV**GA**G**K**T-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_2, a conformation expected to be found in low CO_2 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-4182**TGGT**GAT**TTATTGGCA**-4198-3′) highly homologous to the consensus sequence (5′-CTGGYAYRNNNNTTGCA-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 ureaplasmal 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 $NiCl_2$), 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 ureaplasmal 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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