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Cloning and Expression of *Staphylococcus saprophyticus* Urease Gene Sequences in *Staphylococcus carnosus* and Contribution of the Enzyme to Virulence

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The urease gene of *Staphylococcus saprophyticus* CCM883 was cloned and expressed in *Staphylococcus carnosus* TM300. In vitro translation of the cloned DNA sequences revealed six polypeptides (of 70, 47, 29, 27, 20, and 17 kilodaltons) that were associated with enzyme activity. Introduction of the cloned genes into a urease-negative mutant of *S. saprophyticus* restored the virulence of this strain, confirming our previous suggestion (S. Gatermann, J. John, and R. Marre, Infect. Immun. 57:110–116, 1989) that this enzyme is a major virulence factor of the organism and contributes mainly to cystopathogenicity.

Bacterial ureases, especially those from gram-negative bacteria of the tribe *Proteeae*, have been implicated in the pathogenesis of pyelonephritis (1, 22, 25, 26, 29) and stone formation (14, 25, 26, 30). Consequently, biochemical properties of these enzymes (2, 16, 17, 30, 32) have been studied in detail. Their ecological role and their contribution to uropathogenticity have been reviewed recently (25, 26). However, genetic studies on bacterial ureases revealed considerable heterogenicity even among strains from the tribe *Proteeae* (16, 30, 32). Cloning of urease gene sequences from *Providencia stuartii* (27, 28) and *Proteus mirabilis* (17, 33) showed that in these bacteria, several polypeptides are involved in the native structures of the enzymes. In contrast, jack bean urease, an enzyme of plant origin, is made up of six identical subunits (6). With all bacterial ureases studied so far, additional polypeptides are necessary for expression of enzyme activity (5, 9, 17, 26, 28, 33).

Staphylococcus saprophyticus is known to be a frequent cause of urinary tract infections in young female outpatients (15). In recent studies, we showed that in *S. saprophyticus* the urease contributes mainly to cystopathogenicity, whereas nephropathogenicity and persistence in the urinary tract are governed by other factors (7, 8). The enzyme has a K_m of 6.64 mM urea and an M_r of 420,000 and is inhibited by acetohydroxamic acid. Thus, the biochemical properties of *S. saprophyticus* urease are all within the range reported for other bacterial ureases (16, 26). In our previous study (7), we used a urease-negative mutant derived by mutagenesis with nitrosoguanidine. Although nitrosoguanidine mutagenesis is a well-established method for generation of mutants and is most often used in situations in which other methods of genetic manipulations are not available, generation of double mutations cannot be excluded. In the present study, therefore, we cloned the enzyme in the gram-positive host *Staphylococcus carnosus* and developed methods for transformation of *S. saprophyticus* with plasmid DNA. The results obtained with these techniques showed evidence for a complex structure of the urease operon in this organism and allowed a reassessment of the contribution of the urease to uropathogenicity.

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MATERIALS AND METHODS

Strains and plasmids. *S. saprophyticus* 9325 and its urease-negative derivative GJ1187 have been described in detail previously (7). The type strain, *S. saprophyticus* CCM883, and the host strain for genetic experiments, *S. carnosus* TM300 (11), were a gift of F. Götz, Lehrstuhl für Mikrobielle Genetik, Tübingen, Federal Republic of Germany. For cloning experiments, we used the vector pCA43 (19), which contains the replicon of pC194 and the arsenate and arsenite resistance genes of *S. xylosus* 267.

Media. Bacteria were grown in P broth (7) or on P agar. When needed, chloramphenicol (10 μg/ml; Sigma, Munich, Federal Republic of Germany), Na_2HAsO_4 (2.5 mg/ml), $NaAsO_2$ (100 μg/ml), or urea (0.2%) was added. Screening for urease production was done on Christensen urea agar (Oxoid, Basingstoke, United Kingdom). Phenotypes of deletion mutants were read after 48 h of incubation of 37°C. Media for production and regeneration of protoplasts were as described previously (11, 12).

Preparation of DNA. For large-scale preparation of plasmid or chromosomal DNA, we used a cleared-lysate technique as described by Götz et al. (13), followed by bouyant-density ultracentrifugation in CsCl-ethidium bromide gradients. Briefly, bacteria were grown for 18 h at 37°C in 1 liter of P broth under selective pressure. Cells were harvested by centrifugation, washed twice in 0.15 M EDTA (pH 8.0), and suspended in 30 ml of NACl buffer (2.5 M NaCl, 50 mM Tris, 50 mM EDTA [pH 7.0]). Lysostaphin (Sigma) was added to a final concentration of 17 μg/ml for *S. saprophyticus* and 8 μg/ml for *S. carnosus*, and cell walls were digested at 37°C. Then, 30 ml of lysis buffer (0.3 M EDTA, 0.4% Brij 58, 0.04% sodium desoxycholate [pH 8.0]) was added, and the mixture was incubated for 1 h on ice. After centrifugation (14,000 × *g* at 4°C for 30 min), DNA was precipitated with polyethylene glycol 6000 and subjected to ultracentrifugation. For small-scale isolation from 30-ml cultures, we used an appropriately scaled-down protocol. Before polyethylene glycol 6000-precipitated DNA from small-scale isolations could be digested with restriction enzymes, it had to be extracted with chloroform (chloroform-isoamyl alcohol, 24:1) and dialyzed against TE (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA) for at least 4 h.

Enzymes and buffers. Restriction endonucleases were ob-

tained from Boehringer GmbH, Mannheim, Federal Republic of Germany, or Pharmacia-LKB, Freiburg, Federal Republic of Germany, and used as recommended by the manufacturers. T4 DNA ligase and calf intestinal alkaline phosphatase were from Boehringer; the Klenow enzyme was from Pharmacia. Ligase and end-filling reactions, as well as alkaline phosphatase treatment of plasmid DNA, were conducted as outlined by Maniatis et al. (23).

Transformation of *S. carnosus* and *S. saprophyticus*. For generation of protoplasts and transformation of *S. carnosus*, we used the method of Chang and Cohen (4) as modified by Götz et al. (11, 12). For *S. saprophyticus*, the protoplasts were generated as described for other coagulase-negative staphylococci (10). *S. saprophyticus* was grown in 20 ml of P broth for 18 h at 37°C with continuous agitation. Cells were harvested and suspended in 20 ml of SMMP (0.8 M sucrose, 0.032 M maleate, 0.032 M $MgCl_2$, 1.4% Penassay broth [Difco antibiotic medium no. 3, 0.5% bovine serum albumin [pH 6.5]). Lysostaphin was added to a final concentration of 12 μg/ml. The mixture was incubated with gentle shaking in a water bath at 37°C until the optical density at 600 nm had dropped to one-third of the initial value. The suspension was centrifuged once to pellet intact bacteria (1,600 × *g* for 10 min) and again to harvest the protoplasts (14,000 × *g* at 18°C for 30 min). Protoplasts were suspended in 2 ml of SMMP. To 0.3 ml of this suspension, 30 μl of plasmid DNA in TE and 2 ml of 40% polyethylene glycol 6000 were added. After incubation for 2 min at room temperature (22°C), 7 ml of SMMP was added, and the protoplasts were harvested and plated on DM3 agar. Phenotypic expression (4) was allowed for 4 h. CY overlay agar (11) containing the appropriate antibiotics was added, and the plates were incubated for 3 to 5 days at 37°C.

Construction of a chromosomal gene bank of *S. saprophyticus* in *S. carnosus*. Chromosomal DNA from *S. saprophyticus* CCM883 was prepared as noted above. The DNA was partially digested with *Sau*3AI, and 7- to 20-kilobase (kb) fragments were prepared by electroelution from Tris-acetate gels (23). These fragments were ligated into the *Bam*HI-digested and alkaline phosphatase-treated vector pCA43 (19). *S. carnosus* protoplasts were transformed with the ligation mixture and selected for resistance to chloramphenicol. Clones resistant to chloramphenicol and arsenite but sensitive to arsenate (19) were screened for urease activity on Christensen urea. Positive reactions were visible after 8 to 16 h of incubation (37°C).

Determination of enzyme activity. Preparation of bacterial homogenates in a Braun homogenizer, determination of urease activity, and determination of K_ms have been described previously (7).

In vitro translation. For in vitro transcription-translation of plasmid DNA, we used a commercially available system (procaryotic DNA directed translation kit; Amersham, Braunschweig, Federal Republic of Germany) with [^{35}S] methionine (specific activity, >30 TBq/mmol; Amersham) as the labeled compound. The protocol specified by the manufacturer was followed, and 1 to 2 μg of DNA was used in one reaction. Translation was allowed for 1 h and was followed by a methionine chase reaction for 5 min. Labeled proteins were separated in sodium dodecyl sulfate–12.5% polyacrylamide gels (20), and activity was recorded by fluorography with 2,5-diphenyloxazole (PPO; Sigma) (21) on X-Omat films (Eastman Kodak Co., Rochester, N.Y.).

Animal experiments. Ascending unobstructed urinary tract infection of rats was induced by instillation of 1.5 ml of a bacterial suspension containing 10^9 organisms per ml as described previously (7, 8). Each group comprised 24 animals. Rats were sacrificed on day 3 after infection; bladders and kidneys were removed, ground, and weighed; and viable counts were measured on cystine-lactose-electrolyte-deficient (CLED) agar (Oxoid). The phenotype (chloramphenicol resistance and urease production) of the bacteria isolated from the animals was checked.

Statistical evaluation. For comparisons among three groups, we used the H test of Kruskal and Wallis (3), since a normal distribution of the data cannot be safely assumed (24). If significant differences ($P < 0.05$) were found, the Nemenyi test (31) was applied to show which groups differed.

RESULTS

Cloning of urease genes. We obtained 5,000 chloramphenicol-resistant clones of *S. carnosus*. Of these, 94% were resistant to arsenite but susceptible to arsenate and hence contained inserted DNA. The sizes of the inserts were between 7 and 15 kb. Three clones were urease positive, with plasmids designated pUL545, pUL1410, and pUL1647. Urease activities of these strains of *S. carnosus* were considerably lower than that of *S. saprophyticus* (0.1 to 0.2 versus 8 to 10 μmol of NH_3 per min per mg), but no relevant differences in expression were found among the urease plasmids. Two plasmids (pUL545 and pUL1647) were chosen for further analysis. The third (pUL1410) differed from pUL545 only in containing a 2.4-kb-longer insert not detectably associated with urease activity.

Characterization of urease plasmids. Restriction endonuclease maps of pUL545 and pUL1647 and those of some derivatives generated by partial digestions and religations are shown in Fig. 1. Plasmid pUL545 contained a ca. 7.5-kb insert, and plasmid pUL1647 contained a 7.7-kb one. The most important difference between the inserts was their different orientation with respect to the vector DNA. Plasmid pUL8949 indicates that the adjacent 1.4-kb *Hin*dIII (containing mostly vector DNA) and the 1.2-kb *Hin*dIII fragments of pUL545 were not required for urease expression, whereas pUL8909 shows that the smallest (0.6-kb) *Hin*dIII fragment was needed. The derivatives of pUL1647 show that the 4.2-kb *Xba*I fragment did not contain sequences needed for urease expression (pUL8902), whereas the 1.4-kb *Xba*I fragment was necessary (pUL8903). The smallest insert expressing urease activity was therefore 5.3 kb. The enzyme activity of this strain (0.12 μmol of NH_3 per min per mg) was within the range seen with the other urease plasmids.

Strains for experimental infections. For our experimental infections we chose the urease plasmid pUL8902. For construction of a plasmid containing the same portion of the vector but no insert, we digested pCA43 with *Bam*HI and *Xba*I, which removes genes coding for arsenate and arsenite resistances (19). Ends were filled with the Klenow enzyme in the presence of all four deoxynucleoside triphosphates and blunt-end ligated. As expected, this treatment destroyed the *Xba*I site and restored the *Bam*HI site. Digestion with *Bam*HI and *Cla*I, which also has a unique site within the vector, yielded two fragments and hence gave the anticipated result (data not shown).

The urease-negative mutant strain of *S. saprophyticus*, GJ1187, was transformed with pUL8902 and pCUL1. We found transformation efficiencies of 10^3 transformants per μg of plasmid DNA with this species. Both plasmids and associated phenotypes were stably maintained as shown by

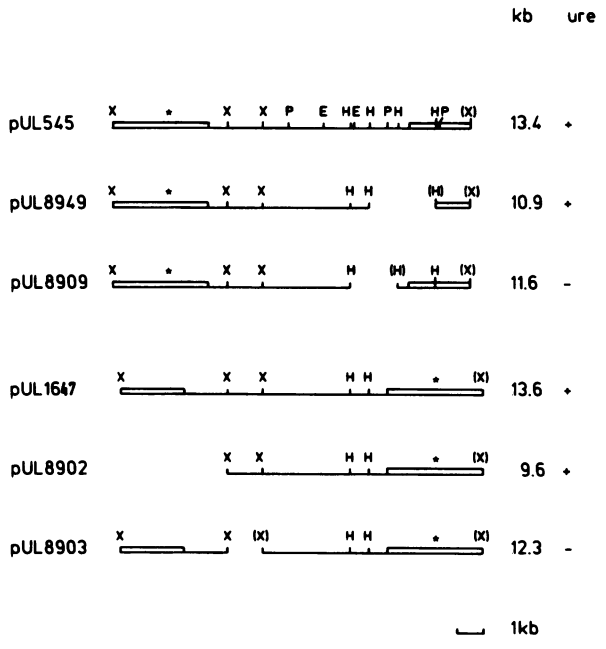

FIG. 1. Restriction maps of two urease plasmids (pUL545 and pUL1647) and some derivatives obtained by partial digestion and religation. ure denotes urease activity of the *S. carnosus* strain harboring this plasmid. Symbols: □, vector DNA of pCA43; *, origin of replication of vector. Abbreviations of restriction endonucleases: X, *Xba*I; H, *Hin*dIII; P, *Pst*I; E, *Eco*RI. Letters in parentheses denote complementing or deleted sites. For clearness, sites not needed for understanding have been omitted from the derivatives.

analysis by repeated subcultures on nonselective media. Enzyme activities in lysates were now 64% of the level found with the donor strain [6.3 μmol of NH_3 per min per mg in GJ1187(pUL8902) versus 9.8 μmol of NH_3 per min per mg in CCM883]. Generation times as tested in P broth did not differ substantially among CCM883 (36 min), 9325 (39 min), GJ1187(pUL8902) (42 min), and GJ1187(pCUL1) (42 min). Also, the K_ms determined from lysates of the urease-positive strains CCM883 (6.64 ± 0.12), 9325 (6.83 ± 0.20), and GJ1187(pUL8902) (6.67 ± 0.25) (mean of three determinations ± standard error of the mean) did not differ significantly as tested by the H test ($P > 0.1$).

Animal experiments. The macroscopic appearance of the bladders of the animals infected with 9325, the parent strain of GJ1187, did not differ from that of bladders infected with GJ1187(pUL8902), the mutant harboring the urease plasmid. With both strains, enlarged bladders containing abscesses and pus in the lumen were seen. In contrast, with the mutant harboring the control plasmid, GJ1187(pCUL1), only slightly enlarged bladders and no pus or abscesses were noted. This situation is reflected by the mean bladder weights, which were 0.21 ± 0.02 g for 9325, 0.28 ± 0.06 g for GJ1187(pUL8902), and 0.12 ± 0.01 g for GJ1187(pCUL1). Statistical analysis by the H test and the Nemenyi test revealed significant differences ($P < 0.05$) between the urease-positive strains and the urease-negative strain, whereas the difference between the two urease-positive strains was not significant. The number of bacteria per gram of bladder tissue was 1.2×10^6 CFU/g for strain 9325, 4.3×10^5 CFU/g for strain GJ1187(pUL8902), and 3.2×10^4 CFU/g for the urease-negative strain GJ1187(pCUL1) (i.e., much smaller) (Fig. 2). Again, differences between the values found for the urease-positive strains did not differ significantly, whereas both differed significantly from the value for the urease-negative strain ($P < 0.05$).

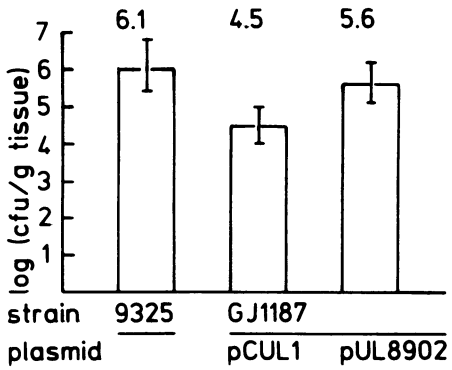

FIG. 2. Bacterial counts in bladders of rats in the three groups. Values are means of log (CFU/gram of bladder) with 95% confidence limits. Differences among strains were significant as analyzed by the H test ($P < 0.01$). The Nemenyi test revealed significant differences ($P < 0.05$) between 9325 and GJ1187(pCUL1) as well as between GJ1187(pUL8902) and GJ1187(pCUL1), whereas the difference between 9325 and GJ1187(pUL8902) was not significant.

Neither kidney weights nor kidney bacterial counts differed significantly among the strains. We found 8×10^3 CFU/g for strain 9325, 1×10^4 CFU/g for GJ1187(pCUL1), and 1×10^4 CFU/g for GJ1187(pUL8902). The kidney weights were 0.79 ± 0.01 g for 9325, 0.78 ± 0.01 g for GJ1187(pCUL1), and 0.79 ± 0.01 g for GJ1187(pUL8902).

Bacteria isolated from the animals always showed the expected phenotype. Thus, colonies isolated from animals infected with GJ1187(pCUL1) were all resistant to chloramphenicol, and those isolated from animals infected with GJ1187(pUL8902) were resistant to chloramphenicol and were urease positive (200 colonies tested for each strain).

In vitro translation of the cloned sequences. Plasmid pUL8902 and, for control purpose, plasmid pCUL1 were transcribed and translated in vitro. A fluorograph of the [^{35}S]methionine-labeled and sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated polypeptides is shown in Fig. 3. Apparently, the urease plasmid pUL8902 encoded several additional proteins when compared with pCUL1, with 17-, 20-, and 29-kilodalton (kDa) polypeptides predominating. Additional polypeptides with apparent molecular masses of 27, 47 and 70 kDa were present in smaller quantities. A faint band of approximately 29 kDa was also present in lane A (pCUL1); however, the 29-kDa band of pUL8902 appeared much more prominent. We did not observe a ca. 57-kDa band reported to be inherent to the kit (33). When transcription-translation products were electrophoresed in 16% acrylamide gels, no insert-encoded polypeptides with molecular masses below 17 kDa could be detected (data not shown).

DISCUSSION

In our study, we cloned gene sequences coding for *S. saprophyticus* urease in the gram-positive host *S. carnosus*. Transfer of these genes into a urease-negative mutant of *S. saprophyticus* allowed analysis of the contribution of the urease to uropathogenicity as well as identification of polypeptides associated with these DNA sequences. In previous studies on the pathogenetic relevance of the enzyme, urease-

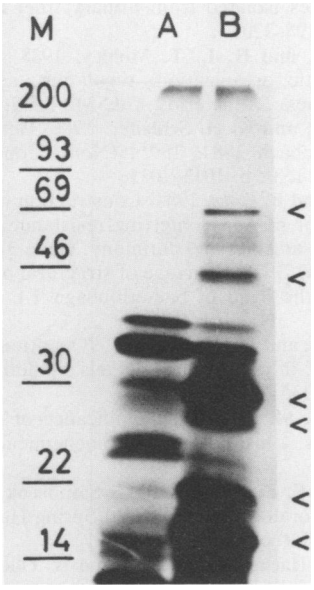

FIG. 3. Fluorograph of the in vitro-translated plasmid DNA of the urease plasmids pCUL1 (A) and pUL8902 (B). About 1 to 2 μg of DNA was used in one reaction. M denotes the position of molecular mass markers (Amersham). Lane B contains six additional polypeptides, with apparent molecular masses of 17, 20, 27, 29, 47, and 70 kDa; their positions are indicated by <.

negative mutants derived by chemical mutagenesis were used (1, 7, 22) or experimentally infected animals were treated with acetohydroxamic acid, an inhibitor of the urease (29). All these studies suggested an important role for the urease in uropathogenicity. Although the quality of mutants generated by chemical mutagenesis may be controlled by careful analysis, double mutations cannot be excluded. When drugs are given to suppress the activity of the enzyme, secondary effects on the bacteria and on the host are possible. Cloning of presumed virulence factors and reintroduction into mutants negative for these factors give the opportunity to rule out some of the pitfalls associated with these experimental approaches.

The cloned urease gene of *S. saprophyticus* was only weakly expressed in *S. carnosus*, the activities being 1/50 to 1/100 of that in the donor strain. As with the donor strain (7), activity was not increased in cultures grown in the presence of urea (data not shown). With cloned ureases of *Proteus mirabilis* (17) and *Providencia stuartii* (28), activity was found to be inducible in the host strain of *Escherichia coli*. About 5.3 kb of DNA was needed for expression of the enzyme, which is close to the sizes reported for *E. coli* (3.2 to 5.8 kb) (5), *Providencia stuartii* (4.4 to 6.0 kb) (28), *Proteus mirabilis* (4.0 to 6.5 kb) (17, 33), and *Morganella morganii* (4.2 to 5.4 kb) (26). With these species, the enzyme structural polypeptides have two (*E. coli*; 27 and 67 kDa) (5) or three (*Proteus mirabilis* and *Providencia stuartii*; 5 to 9, 8 to 10, and 68 to 73 kDa) subunits (17), with additional polypeptides needed for regulation and, presumably, for nickel transport. For ureases from gram-positive organisms, usually less complex structures have been described (26). By using in vitro transcription-translation, we found six labeled polypeptides in addition to those encoded by the control plasmid (containing the same vector sequences as the urease plasmid). These polypeptides had apparent molecular masses of 17, 20, 27, 29, 47, and 70 kDa. Since a faint 29 kDa band was observed in the control plasmid, the possibility that this polypeptide is of vector origin cannot be ruled out; however, the 29-kDa band was much more prominent in the urease plasmid, whereas other polypeptides of the vector were obviously present in smaller amounts only. Therefore, in pUL8902 there may be two polypeptides in the 29-kDa region with only slightly differing molecular masses. The occurrence of a 47-kDa polypeptide is very interesting, since a polypeptide of similar size (43 kDa) has been described only for *Klebsiella pneumoniae* (9), whereas with the other species the additional proteins were always smaller than 30 kDa. Our findings of 70- and 47-kDa polypeptides associated with enzyme activity are also in agreement with our previous observations (7) that enzyme bands eluted from native gels gave rise to an approximately 70-kDa polypeptide on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. On immunoblots developed with antisera from infected animals, however, an additional 48-kDa band was clearly visible. Together with our present data, this suggests that the native structure of the enzyme may involve the 70- and 47-kDa polypeptides.

When the urease-negative strain of *S. saprophyticus*, GJ1187, was transformed with the urease plasmid pUL8902, urease activity in lysates was considerably higher again (6.2 versus 0.1 μmol of NH_3 per min per mg). The very low activity in the *S. carnosus* host strain may be due to the relatively distant relationship of the two species (18) or may indicate the presence of additional regulatory DNA sequences in *S. saprophyticus*.

Experimental infection with the wild-type strain 9325 (urease positive), GJ1187(pCUL1) (the urease-negative mutant containing vector DNA only), and GJ1187(pUL8902) (the urease-negative mutant with the urease plasmid) showed similar signs of inflammation in the bladders of rats infected with the urease-positive strains, and the bacterial counts per gram of organ did not differ significantly between these strains. In contrast, bladders of animals infected with the urease-negative strain showed significantly lower bladder weights, indicating less inflammation, and the bacterial counts differed significantly from those of both other groups. Interestingly, as reported previously (7), kidney weights and kidney bacterial counts did not differ among the three groups.

In our study, we found new evidence for the pathogenetic relevance of *S. saprophyticus* urease by cloning the virulence factor in a gram-positive host and reintroducing the cloned gene into a urease-negative mutant of *S. saprophyticus*. We therefore confirmed our previous suggestion that the urease contributes mainly to cystopathogenicity in this organism. Moreover, our study showed that genetic manipulations for the study of virulence factors are possible in this species and therefore that the molecular Koch's postulates may be fulfilled. In addition, in vitro translation of the cloned DNA identified six polypeptides that were encoded by the urease gene sequences. This shows that in the gram-positive organism *S. saprophyticus*, several polypeptides are needed for expression of urease activity. Our work also allows a study of the possible relationship of urease genes between gram-positive and gram-negative species and offers an opportunity to assess the function of polypeptides associated with enzyme activity.

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