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Urea-Hydrolyzing Activity of a T-Strain Mycoplasma: *Ureaplasma urealyticum*

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The urea-hydrolyzing activity of a T-strain mycoplasma was studied in experiments using whole cells and cell-free enzyme preparations by measuring the release of $^{14}CO_2$ from [^{14}C]urea. Under the conditions used, the urea concentration optimum is approximately 5.6×10^{-3} M urea. The activity is soluble and not membrane bound. It is stable at −70 C for several weeks but is more labile at higher temperatures. The pH optimum is between 5.0 and 6.0. The effect of several inhibitors on the activity was tested and revealed similarities, as well as differences, between T-strain mycoplasma urease activity and the urease activity of other organisms and plants.

The ability of T-strain mycoplasmas to hydrolyze urea was first reported by Shepard (19) and independently by Purcell et al. (16) and described in more detail soon afterwards (6, 20). This one metabolic capability of the T-strain mycoplasmas is now thought to differentiate them sufficiently from other mycoplasmas so that human T-strains have been recently classified as a new and separate genus (*Ureaplasma*) within the class *Mollicutes* (21). Although "production of urease, and therefore ... ability to hydrolyse urea" (21) is given as the major differentiating characteristic for T-strains, there is no published evidence that these organisms possess or produce a single specific enzyme that hydrolyzes urea, and little is known about this activity in T-strain mycoplasmas. Perhaps this lack of information reflects the difficulties in working with these organisms. They grow to comparatively low maximum titers and die very rapidly (21). This and their small size make it difficult to obtain reasonable amounts of material from actively growing cultures for enzyme study. These initial studies of the *Ureaplasma* urease activity, then, are not yet as elegant as some of the enzymology that has been done in recent years, but it is a beginning. Some work has already been reported by others.

In 1973, carbamate ion was reported to be an intermediate in a two-step urealytic reaction by T-strain enzyme preparations (P. K. Joo, L. Marnell, and P. J. VanDemark, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, M41, p. 80), but further supporting evidence has not been forthcoming. Prior to that, Ford et al. (5) showed that about 95% of the $^{14}CO_2$ released from [^{14}C]urea used as substrate for T-mycoplasmas could be recovered and was not incorporated by the organism. Thus, a physiological function for T-strain urealytic activity is not at all established. Nor can it be said at this time that urea is "required for best growth or multiplication of T-mycoplasmas" (21) since it has been shown that laboratory-adapted strains grow well without urea added to the medium either directly or via the serum or protein medium enrichment used (11, 13, 14). This observation has now been extended to clinical isolates as well (Masover and Hayflick, unpublished data). Thus, whereas the presence of a T-strain urealytic capability is well established, the enzyme activity responsible for it is not well understood. We have, therefore, undertaken to study this activity, and we report here some observations relevant to urea hydrolysis by T-strain mycoplasmas. These observations extend what is presently known about this differentiating characteristic of T-strain mycoplasmas.

MATERIALS AND METHODS

T-strains and media. *U. urealyticum* (formerly T-strain 960) was originally supplied by M. C. Shepard (Camp Lejeune, N. C.) after being cloned eight times. It was passaged in our laboratory more than 30 times in medium without added urea, as previously described (12).

Our routine growth medium for T-strains has also been previously described (11, 12). It differs from mycoplasma broth medium (7) in that: (i) 10% (vol/vol) unheated horse serum is used instead of 20% (vol/vol) of this supplement; (ii) 1% (vol/vol) of

10×-concentrated phosphate-buffered saline is added to give a final concentration of 0.1× phosphate-buffered saline; (iii) urea is added to give a final concentration of 0.1% (wt/vol) (glucose is omitted); and (iv) the pH is adjusted to 6.5.

Some CO_2 trapping experiments were done in unsupplemented Eagle basal medium (BME).

T-strains were quantitated by the method previously described (14) and expressed as 50% color change units (CCU_{50}) per milliliter.

Digitonin lysis. Digitonin lysis of T-strains was accomplished by the method of Rottem and Razin (18) using 20 μg of digitonin per ml of 0.25 M NaCl solution.

Protein. Protein was estimated by the method of Lowry et al. (10).

CO_2 trapping. CO_2 trapping was done in 50-ml Erlenmeyer flasks that had been permanently fitted with center wells. Small containers were made to fit these center wells by breaking off the tops of glass serological test tubes (13 by 100 mm) and using the bottom 20 mm of the tube. After thorough washing and drying of the flasks, 4.0 ml of medium containing 50% more than the desired final concentration of urea was added to the flask in the urea outside of the center well. A container, placed in the center well, was filled with 0.5 ml of hyamine hydroxide, and the flask was sealed with a rubber serum stopper. A slight vacuum was then created in each flask by means of a suction pump fitted with appropriate tubing and a hypodermic needle. The outer portion of each flask received, by injection, 1.0 ml of deionized water (pH 7.0) containing 0.1 μCi of [^{14}C]urea and 1.0 ml of either enzyme preparation in 0.25 M NaCl or late-log-phase T-960 organisms in the T-strain growth medium. It is recognized that in this latter case a small and undetermined amount of urea is added with the organisms. In the case in which cell-free enzyme preparations are used, the total urea content is known, including the amount present in the horse serum medium supplement. This was determined by analysis of deproteinized complete medium by ion exchange chromatography on Durrum DC-6A cation exchange resin (Durrum Chemical Co., Palo Alto, Calif.) as previously described (11). Urea hydrolysis was stopped after a specified time (usually 1 h) by injection of 3.0 N H_2SO_4 (1.0 ml/flask) and was allowed to incubate with either occasional or continuous swirling of the flasks for an additional 2 h. This served to release CO_2 from the medium, and it facilitated trapping the CO_2 in the hyamine hydroxide. No difference in results was observable between flasks swirled continuously or only occasionally (every 10 or 15 min). After this 2-h period, the entire container with hyamine hydroxide and trapped carbon dioxide, as well as a sample of the medium outside the well (containing unhydrolyzed [^{14}C]urea), was removed for liquid scintillation counting.

The efficiency of ^{14}C counting on the instrument used was always about 85%. Efficiency of trapping in the CO_2 trapping system was determined by adding excess (25 mg/ml) crystalline jack bean urease (Nutritional Biochemicals Corp., Cleveland, Ohio) to medium containing 0.1 μCi of [^{14}C]urea and varying amounts of added nonradioactive urea as a carrier (0 to 0.075% [wt/vol]). Under these conditions, 100% of the urea was hydrolyzed, as evidenced by the absence of radioactivity in the medium outside the wells. The counts trapped by the hyamine hydroxide amounted to 95% of those that were originally added to the flask. This, then, represented the efficiency of CO_2 trapping. For all following experiments, radioactivity in both the CO_2 trap and the reaction medium was measured and, in all cases, the summed radioactivity was ±5% of the amount expected, considering the efficiencies determined.

Liquid scintillation counting. Liquid scintillation counting was done using an Isocap 300 liquid scintillation system (Nuclear-Chicago Corp., Arlington Heights, Ill.). Samples were counted at room temperature after 2 or 3 days equilibration in the dark. Glass scintillation vials were used, and samples were added to 10.0 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) scintillation solution.

Radioisotopes and reagents. [^{14}C]urea with a specific activity of 54.4 mCi/mmol (877 μCi/mg) was purchased from Amersham/Searle (Arlington Heights, Ill.). Acetohydroxamic acid (AHA) was obtained from the Aldrich Chemical Co., San Leandro, Calif. Hydroxyurea, thiourea, and digitonin were purchased from Calbiochem, La Jolla, Calif. The gold preparation used was Myochrisine (Merck & Co., Inc., Rahway, N. J.) brand of sodium aurothiomalate in sterile aqueous solution (50 mg/ml).

RESULTS

Figure 1 shows the results of several representative experiments in which we attempted to select a late-log-phase culture of the organism as the source of enzyme and where the reaction medium was the complete T-strain growth medium. It can be seen that a saturation curve is generated and that the maximum velocity for CO_2 release occurs at a substrate concentration of approximately 5.6×10^{-3} M urea (0.033%, wt/vol) in all cases. When Lineweaver-Burk plots (reciprocal of reaction velocity versus reciprocal of substrate concentration) are made with these same results, a K_m of 2.78×10^{-3} M (0.0167%) urea is observed. However, the amount of activity was not always correlated with the number of viable organisms per flask. This is not surprising since we also observed (not shown) that organisms taken from cultures in the early part of the death phase gave similar activity and the accuracy of the CCU_{50} determinations can vary by about a factor of 2, whereas the variability of the urea hydrolysis determinations is usually ±10%. In a majority of experiments, the activity could be related to the number of viable organisms.

It was of immediate interest to determine whether the urea-hydrolyzing activity in these

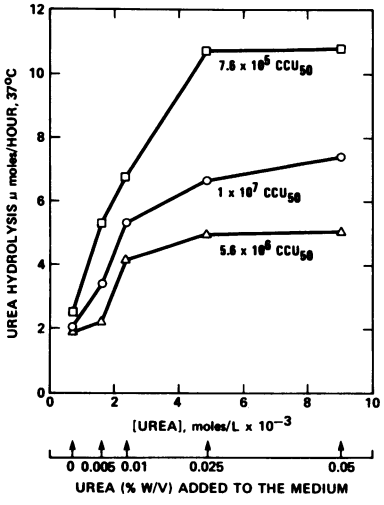

FIG. 1. *Urea hydrolysis by whole cells of T-strain 960 in complete medium containing 10% (vol/vol) horse serum (500 μg of urea/ml) and added urea. Each point is the mean for duplicate flasks, prepared as described in the text and containing a total volume of 6.0 ml. The range in all cases was 10% or less. Each symbol represents a separate experiment.*

organisms could be demonstrated in a cell-free system since that would allow for better characterization of the activity. Batches of log-phase T-960 were harvested by centrifugation at 27,000 × *g* for 15 min and washed twice in 0.25 M NaCl prior to lysis with digitonin (18). Some urea-hydrolyzing activity was found associated with the membrane pellet immediately after digitonin lysis, but this was completely removed by the first wash of the membrane portion of the lysate. From this we conclude that the T-strain mycoplasma urease activity is soluble and not membrane bound.

The activity observed in several different soluble preparations from digitonin-lysed T-960 was reasonably reproducible when tested under similar conditions. For example, the activity measured in BME, containing 0.025% urea at pH 5.0 and 37 C, was between 0.29 and 0.36 μmol of urea hydrolyzed per h per μg of protein for four separate preparations.

When the lysate was stored at −20 C, we found that its activity was lost in 2 to 3 weeks. However, if stored at −70 C, most of the activity remained for at least 5 weeks (see Fig. 3).

Assay of total protein from whole washed T-strains agreed well with the sum of protein in soluble (supernatant fluid) and membrane (pellet) fractions of digitonin-lysed T-strains, indicating that little or no protein was lost in the lysis procedure. A typical 2-liter preparation containing approximately 10^6 CCU_{50} of T-960 per ml yielded 600 μg of soluble protein and 25 times that much protein in the membrane pellet. This high ratio of membrane to soluble protein suggests that medium proteins are being measured in the washed membrane preparation. The soluble portion was dissolved in 0.25 M NaCl (pH 7.2) to give a final concentration that varied between 16 and 48 μg of protein per ml of solution, most preparations being between 25 and 30 μg of protein per ml.

Figure 2 shows the urea-hydrolyzing activity of the cell-free soluble enzyme preparation in either whole T-strain medium or in unsupplemented BME plus 0.1% (wt/vol) ethylenediaminetetraacetic acid (EDTA) (2.8×10^{-3} M). Maximum and half-maximum velocities for production of $^{14}CO_2$ from [^{14}C]urea occur at approximately the same urea concentration as was observed with intact cells. It is, therefore, possible to study this activity in a cell-free system.

Since all of the requirements for optimum enzyme activity were not known and preliminary experiments with a tris(hydroxymethyl)-aminomethane-maleate buffer and with two different phosphate buffers gave poor results, the more complex, but still defined, BME was chosen as the reaction medium for initial experiments with the cell-free, soluble T-strain urease activity. The pH optimum was tested first, and the results of representative experiments are shown in Fig. 3. The pH optimum is between pH 5.0 and 6.0 for these conditions (BME plus EDTA medium at 35 to 37 C) and is seen to decrease sharply above and below the optimum range.

The assay is buffered only to the extent that

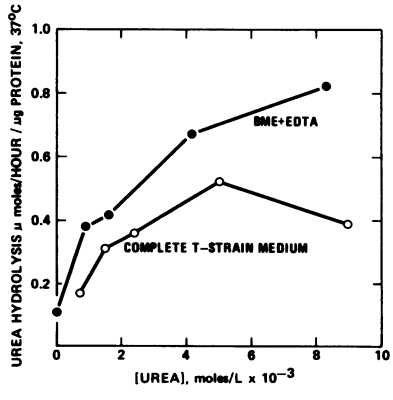

FIG. 2. *Urea hydrolysis by the soluble fraction of digitonin-lysed T-strain 960. Symbols: ●, BME plus 0.1% EDTA; ○, complete T-strain medium.*

the BME is buffered. This allows for some pH change during the reactions in which the activity or the urea concentrations are very high. Under the conditions usually used (initial pH, 5.0, and initial urea concentration, 0.025%), the pH change is moderate (<1 pH unit) and does not appear to interfere with the reaction. The two experiments shown in Fig. 3 were done using the same batch of enzyme but, in one case (closed circles), the experiment was done immediately after digitonin lysis of the cells. In the second experiment (open circles), the enzyme preparation had been frozen at −70 C for 5 weeks. The loss of activity can be seen to be relatively small during this time.

Although the pH optimum was found to be similar in BME either containing EDTA or without it (not shown), the total activity is greater when EDTA is present. This suggests that the T-strain urease activity is sensitive to cations which would be chelated by EDTA. This was shown to be the case in that the maximum activity in BME with EDTA was about double the maximum activity in BME without EDTA. Addition of 1 mM each of calcium and magnesium ion to the BME with EDTA reduced the activity to the lower levels again, as would be expected, since the added amount of calcium and magnesium, along with the calcium and magnesium already present in BME, is sufficient to overcome the chelating capacity of the amount of EDTA used. Therefore, for study of the effects of various inhibitors of the T-strain urease activity, EDTA was included in the reaction medium in most cases. A concentration of urea (0.025%) and pH of 5.0 were chosen, based on the previously determined optima.

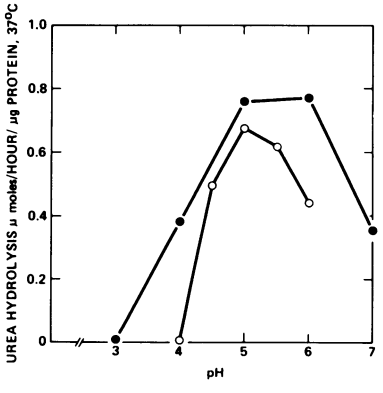

FIG. 3. *Determination of pH optimum for urea hydrolysis by soluble T-strain 960 urease activity. Urea hydrolysis was measured as described in the text. The reaction medium for these experiments was BME plus 0.1% EDTA and 0.025% urea. Complete reaction flasks were incubated for 1 h at 37 C and were gently agitated at intervals during the incubation period. Symbols:* ●, *freshly prepared T-strain 960 urease activity;* ○, *same enzyme preparation after 5 weeks of storage at −70 C.*

The effect of several substances that might be expected to have some effect on the T-strain urealytic activity was tested, and results of several experiments of this type are summarized in Table 1. It can be seen that the soluble urease activity was not at all inhibited by 1:2,000 thallium acetate or by the lower concentration of acetohydroxamic acid (AHA) although this amount of AHA has been shown to cause complete inhibition of much larger amounts of crystalline jack bean urease (3). Gold (aurothiomalate) caused complete inhibition. Pretreatment of the enzyme preparation by 100 C heat for 5 min also eliminated the activity completely. Hydroxyurea at double the urea concentration (on a weight basis) caused almost complete inhibition of the urease activity. On the other hand, thiourea at the same concentration as hydroxyurea did not have as pronounced an effect on the release of $^{14}CO_2$.

Increasing amounts of AHA caused decreasing activity, such that the AHA gave 50% inhibition at a concentration of about 3.7×10^{-4} M. The weakly inhibitory effect of small amounts of phosphate is seen also. Other ions and inhibitors (not shown) were also tested in the BME reaction medium, both with and without EDTA. It was found, for example, that ammonium ion slightly enhanced the activity at concentrations of 100 and 250 μg/ml in the presence of EDTA and also at 500 μg/ml in the absence of EDTA. We cannot say whether this is an effect of ammonium ion specifically or of a particular concentration of monovalent cation. This point cannot be readily elucidated in a reaction medium such as BME, which already contains considerable quantities of sodium and potassium. It must be studied in a different and simpler reaction mixture, which may be better controlled with respect to the monovalent cations. Nevertheless, it is clear that the product of this enzyme reaction does not inhibit the enzyme activity, as is the case with many enzymes including some ureases (1).

DISCUSSION

The urealytic activity of *U. urealyticum* was assessed by measuring the accumulation of CO_2, a product of the activity. Urea carbon has been shown previously (5) to be completely recoverable as CO_2 and not incorporated by the

TABLE 1. *Effect of inhibitors on T-strain urease activity*

Inhibitor added	Amt	Activity (% of control)[a]	
		-EDTA	+EDTA (0.1%)
AHA	5×10^{-3} M	NT[b]	3.8
AHA	1×10^{-3} M	29.2	17.9
AHA	5×10^{-4} M	NT	37.1
AHA	1×10^{-4} M	NT	91.4
Thallium acetate	1:2,000 (1.9×10^{-3} M)	96.2	102.6
Gold (aurothiomalate)	1,100 μg/ml (5.6×10^{-3} M)	0	NT
Thiourea	0.05% (wt/vol) (6.6×10^{-3} M)	43.1	69.6
Hydroxyurea	0.05% (wt/vol) (6.6×10^{-3} M)	NT	10.0
Phosphate-buffered saline	1× (in addition to BME phosphates)	NT	68.0

[a] All experiments were done in BME (pH 5.0, 37 C) with 0.025% (wt/vol) urea. Each reaction flask received 1.0 ml of enzyme solution containing 25 to 30 μg of protein per ml and 0.1 μCi of [^{14}C]urea, as described in the text. Results were calculated as micromoles of urea hydrolyzed per microgram of protein.

[b] NT, Not tested.

organism. It was possible to demonstrate that the urea concentration at which optimum hydrolysis and CO_2 production occurred in both whole cells and in cell-free preparations was 5.6×10^{-3} M, which is 0.033% (wt/vol). This is approximately half the amount generally recommended for T-strain medium formulations to achieve optimum growth (6, 20). For example, a medium with 0.05% urea added plus 20% (vol/vol) of a horse serum containing 400 to 600 μg of urea per ml (13, 20) would have a final urea concentration of approximately 0.06%. This amount of urea in the growth medium will give rise to the expected growth, without sacrificing the readily perceptible color change most commonly used as a major criterion for recognition of these organisms. On the other hand, a total urea concentration of 0.03% (approximately 0.02% added urea) in medium containing 20% horse serum would produce a comparatively weak color change. This would be due to reduced total ammonia production from the reduced amount of urea initially present and also from the high buffer capacity of the protein-enriched medium. In both cases, the urea present in the medium would be completely hydrolyzed independently of the growth rate or the optimum growth potential of the organisms (11). Thus, the T-strains we tested appear to be indifferent to urea concentrations greater than 0.033%, but mycoplasmologists may prefer the higher urea concentrations, anyway, to facilitate detection of the organisms.

We have not, as yet, tested fresh clinical isolates for optimal urea concentration in this manner, but we have collected many T-strain isolates in medium without added urea (<5 μg/ml) (Masover and Hayflick, unpublished data). We know of no published evidence that there are differences in hydrolysis of urea by laboratory-adapted T-strains compared with fresh clinical isolates.

We also found that the activity was soluble, rather than membrane bound, and could be stored for at least 5 weeks without great loss of activity. This allowed for the first study of this differentiating activity of ureaplasmas in a cell-free preparation containing both undefined mycoplasma broth medium and a defined reaction medium (BME with EDTA). In this manner, the pH optimum for the activity in a cell-free preparation was determined and found to agree with the optimum pH (5.0 to 6.0) for growth or urea hydrolysis by whole cells (20, 21). Other urease activities are reported to have higher pH optima (8, 9, 23)

The experiments with inhibitors allow us to make initial comparisons between the effect of a given substance on: (i) the cell-free urealytic activity of ureaplasmas, (ii) the reported effect on growth of the whole organism, and (iii) the reported effects on urealytic activities from other sources. For example, the urease activity of *Proteus vulgaris* has been reported to be soluble (15) and inhibited by AHA: 100% inhibition by 500 μg of AHA per ml and 50% inhibition by 15 to 30 μg of AHA per ml. This is very similar to the AHA inhibitory concentrations we have observed using the T-strain soluble urease activity: >95% inhibition by 5×10^{-3} M (375 μg/ml) and 50% inhibition with 3.7×10^{-4} M (28 μg/ml). This same report (15) shows that *P. vulgaris* urease activity was 100% inhibited by 62 μg of thiourea per ml. By comparison, we found that the T-strain urease activity was approximately 50% inhibited by 500 μg of this

inhibitor per ml. This suggests that there is some difference in the sensitivity of these two urease activities to thiourea. By extension, these observations suggest that there may be some difference in the enzymes involved in the urea hydrolysis. Thiourea was also reported to inhibit the urease activity in intact cells and cell-free extracts from *Corynebacterium renale* (9). In this case, if the thiourea was substituted on the sulfur atom, the inhibitory activity was decreased, but N substitution of the analogue had no effect on the inhibitory activity. This suggests that the *C. renale* urease activity recognizes the carbonyl portion of the substrate preferentially to the urea nitrogens. Conversely, the T-strain urease activity was inhibited by the N-substituted hydroxyurea, whereas jack bean urease can use both hydroxyurea and dihydroxyurea as substrates (2, 3, 4). The hydroxyurea is an inhibitor of the jack bean urease, even though it is a substrate, whereas the methyl-substituted hydroxyurea analogue AHA is an inhibitor but is not hydrolyzed (3, 4). The T-strain urease activity has not been tested yet for its ability to hydrolyze either hydroxyurea or hydroxamic acids.

We have observed that the T-strain urease activity is slightly sensitive to phosphate, and this has also been observed in whole cells (20). Jack bean urease has been reported to be slightly inhibited by phosphate ions, but phosphate buffers are used routinely anyway in urea analysis by crystalline urease preparations (1). *Corynebacterium* and *Bacillus* urease activities are also assayed in phosphate buffer (8, 9). There does not appear to be either agreement or understanding regarding the effect of phosphate on urease activities.

As a final example, we observed that the T-strain urealytic activity was slightly enhanced by ammonium ion, as is also the case for the energy-requiring urea amidolyase of *Candida utilis* (17) and some other microbial species. Ammonium ion is reported to have some inhibitory effect on jack bean urease (1). We cannot say that the enhancement of the T-strain urease activity is specifically due to ammonium ion as opposed to another monovalent cation since our reaction medium (BME plus EDTA) contains large quantities of K^+ and Na^+. It is clear, however, that NH_4^+ does not inhibit the activity. This and other questions relevant to the requirements of T-strain urealytic activity must await a simpler reaction system. Preliminary experiments with several buffers suggest that *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer may serve well for this purpose. Tris(hydroxymethyl)aminomethane-maleate, veronal acetate, 2-(*N*-morpholino)ethanesulfonic acid (MES), and phosphate buffers were either ineffective or less effective than HEPES in our assay of the T-strain urease activity. Once a buffer is selected, it will be of interest to study the effect of bicarbonate ion also, since CO_2 enhances growth of T-strains on agar (21) and is also a requirement of urea amidolyases (17).

The term "urease," as usually used, is a generic term for a number of enzyme species that are able to cleave the carbon-nitrogen bonds of urea with production of ammonia and CO_2. There is currently no reason to believe that the T-strain mycoplasma urealytic capability results from a single enzyme comparable to the urease activities that have been isolated and purified from other microbial species or plants. Nor is there evidence available now to relate T-strain urease activity to either production or consumption of energy or to any physiological function in the organism. The physiological function of the ureases of other organisms or plants is not established either, and perhaps this is the point of major interest. This activity is a property of the first enzyme ever to be prepared in crystalline form 50 years ago by Sumner (22). It has now been described in hundreds of simpler life forms (prokaryotic and eukaryotic), and the hydrolysis of urea is now known to be accomplished by different protein species having different requirements. However, the function of this widespread activity is not understood for any "urease-positive" plant or microorganism.

It is of some interest that a simple organism with a limited genome, such as a mycoplasma, should be willing or able to invest in this activity although it does not appear to be required for the multiplication of the organism (11, 12, 13). The T-strain mycoplasmas are undoubtedly the simplest organisms known to possess this activity and, as such, perhaps they will ultimately provide a tool for understanding a physiological function of urease activities in general.

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