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NOTES

Cultivation of *Ureaplasma urealyticum* in Continuous Culture

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Continuous culture of *Ureaplasma urealyticum* is reported with a steady-state cell biomass of $>10^6$ cells per ml. Thus, large cell numbers can be easily obtained; in addition, the system provides a powerful means for exploring what nutrient(s) limits the growth yield of this organism. Urea is shown not to be the growth-limiting nutrient in conventional media, although when provided in excess it appears to be completely hydrolyzed.

The members of the class *Mollicutes* (mycoplasmas) are of considerable biological, biochemical, and medical interest in that they are the simplest organisms known which are capable of an independent existence and are involved in human and animal pathogenicity (4, 13). Mycoplasmas included in the genus *Ureaplasma* possess urease activity and can consequently hydrolyze urea (18). This capability is widespread in the biological world, but its physiological function remains uncertain in most cases (15); *Ureaplasma*, being the simplest organism possessing this activity, may prove to be a convenient system for exploring this function.

The study of these organisms is hampered, however, by low growth yields and the nonavailability of a defined growth medium. The problem is especially acute in the case of *Ureaplasma*, which attains despairingly low titers and loses viability rapidly in conventional batch cultures (5, 9). We describe here cultivation of *Ureaplasma urealyticum* in a chemostat under steady-state conditions. This has enabled us to maintain logarithmic growth of this organism under relatively defined conditions over long time periods, thus affording a facile way of obtaining large cell numbers. More importantly, it provides a convenient means of exploring what factor(s) limits the growth of this organism in conventional media. We present here evidence that shows unequivocally that urea is not the growth-limiting nutrient for this organism in conventional media, contrary to frequent assertions made to this effect in the literature (1, 4, 5, 17).

Our laboratory strain (T-960) of *U. urealyticum*, serotype VIII, originally supplied by M. C. Shepard (Camp Lejeune, N. C.), was used in this study. Separate aliquots of a single pool of cells, which had been frozen (−70°C) in broth at late logarithmic phase, served as inocula for individual experiments; these were thawed, suitably diluted, and grown overnight in 2 ml of medium. The medium used was similar to that previously described (6, 7), except that Difco yeast extract (0.75%, wt/vol) was employed instead of the freshly prepared yeast extract described by Hayflick (3); only 5% (vol/vol) whole unheated horse serum (Pacific Biological Co.) was used; and the urea concentration was varied as described in the text. The contribution of urea by the added serum was negligible (<2%). In some experiments horse serum was replaced by calf serum.

The chemostat employed was a C-30 Bioflo unit (New Brunswick Scientific Co.) with a working volume (V) of 350 ml. The growth vessel was stirred at 200 rpm; higher stirring speeds caused excessive foaming of the medium. Approximately 0.2 liter of air per min was passed through the culture, and the pH was maintained at 6.9 ± 0.1 using a New Brunswick pH stat, which automatically pumped sterile 0.5 N HCl solution into the culture to neutralize the alkali produced; the amount of acid added during each experiment was recorded. Other features of the chemostat were as previously described (2, 12).

The inoculum size was adjusted so as to give an initial concentration approximately 10^4 cells per ml in the chemostat vessel. After overnight incubation as batch culture (i.e., without medium flow), the medium pump was adjusted to give a fixed flow rate (F) of the fresh medium into the chemostat vessel; F/V equals the dilution rate (D). During the experiments, approxi-

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mately 5-ml samples were removed from the chemostat vessel, and the cell titer was determined as 50% color change units, as described previously (8).

Steady-state growth of *U. urealyticum* was obtained at three *D* values (Fig. 1), since at each of these values the cell biomass in the culture vessel and the rate of urea hydrolysis remained roughly constant through several volume changes. At $D = 0.1$ and $0.15\ h^{-1}$, the steady-state cell number in the culture vessel was approximately $10^7\ ml^{-1}$ and the rate of acid addition equaled that expected if all the urea added to the growth chamber were completely hydrolyzed. At $D = 0.05\ h^{-1}$, the steady-state cell biomass decreased, presumably because of the energy of maintenance requirement (14), and the rate of acid addition was somewhat less than expected from complete hydrolysis of the urea added. In a separate series of experiments, substitution of horse serum by calf serum in the medium gave essentially identical results at $D = 0.10$ and $0.15\ h^{-1}$, i.e., identical cell titers and rates of urea hydrolysis were obtained. Thus, both horse and calf sera support the growth of this organism to a similar extent, contrary to the usual recommendation of horse serum as the superior native protein enrichment for *Ureaplasma* (16, 18).

Such steady-state cultivation ensures a constant supply of healthy, logarithmically growing cells. It can be calculated that, for instance, at $D = 0.15\ h^{-1}$, 1.2×10^{10} cells are produced and stored in the product reservoir every 24 h; by simply placing the product reservoir in a refrigerator (11) and harvesting at regular intervals, these cells can be obtained for experimental purposes. Since the μ_{max} of this organism is approximately $0.4\ h^{-1}$, it is evident that much greater rates of cell production can be obtained by employing higher *D* values.

Continuous culture affords a powerful means for exploring what nutrient(s) is limiting the growth of an organism in a given medium (10). Since the steady-state cell biomass in the culture vessel at a given *D* value is determined by the concentration of the limiting nutrient in the inflow medium (11), it follows that changes in cell biomass following changes in the concentration of a specific nutrient in the inflow medium would identify that nutrient as growth limiting. The urea concentration in the inflow medium used in these studies was 10 mM. To determine if urea at this concentration was growth limiting, we grew the organism for several volume changes at a fixed *D* ($0.1\ h^{-1}$) but using a lower (5 mM) and a higher (20 mM) urea concentration in the inflow medium. The steady-state cell biomass in the culture vessel remained unaffected by these changes in the urea concentration (Fig. 2), indicating clearly that urea, when present at 5 mM concentration, is not the growth-limiting nutrient for *U. urealyticum* in the medium employed. Nevertheless, when urea was provided at 10 and 20 mM concentration in the inflow medium, it appeared to be completely hydrolyzed, as indicated by the rate of acid addition. It follows that increased urea hydrol-

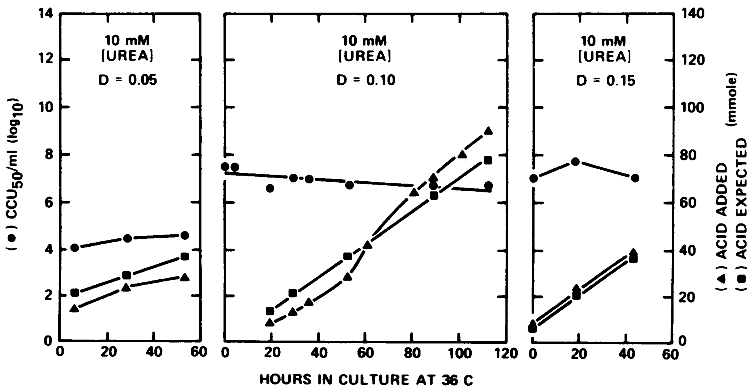

FIG. 1. *Growth of U. urealyticum in continuous culture at various dilution rates. The urea concentration of the medium was 10 mM. See text for experimental procedure. Samples for cell quantitation as 50% color change units (CCU_{50}) per milliliter were taken as shown* (●). (▲) *Amount of acid added automatically to maintain the pH at 6.9.* (■) *Amount of acid expected to be used assuming that: (i) the urea added (10mM at 17.5 [D = 0.05], 35 [D = 0.10], or 51.5 [D = 0.15] ml h^{-1}) was hydrolyzed completely to NH_3 and CO_2 so that each mole of urea introduced would yield 2 mol of NH_3; (ii) NH_3 from urea was the only source of alkali and there was no concomitant production of acid; and (iii) the buffer capacity of the medium and the small amount of urea in the 5% added horse serum did not appreciably affect this estimate. The three separate panels represent a single run during which only the dilution rate (D) was altered.*

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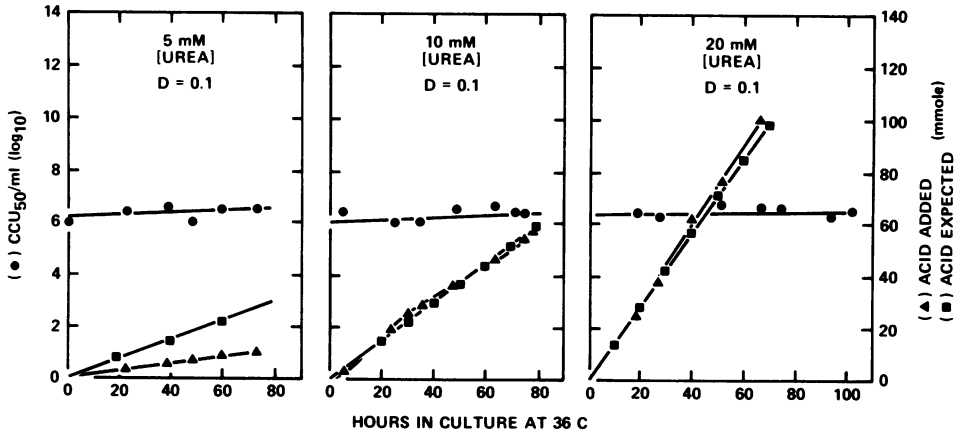

FIG. 2. *Growth of Ureaplasma urealyticum in continuous culture at a fixed dilution rate (0.10 h^{-1}) but with varying urea concentrations in the inflow medium. The chemostat was inoculated and run as described in the text. Symbols: (●) growth; (▲) acid automatically added to maintain the pH at 6.9 ± 0.1; (■) expected acid addition (see legend to Fig. 1).*

ysis at higher urea concentration in the inflow medium has no apparent physiological significance for the organism. Parenthetically, since urease is an intracellular enzyme in this organism (9) and is inactivated by trace amounts of metals (15), it is highly unlikely that the increased urea hydrolysis occurred extracellularly. It must be stressed that these results were obtained with a single laboratory strain, and their general validity should be checked using other, preferably freshly isolated strains.

Further work to attempt to identify the limiting nutrient(s) in the medium of *U. urealyticum*, using chemostat cultures of this organism, is currently in progress.

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