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ATP Measurements Obtained by Luminometry Provide Rapid Estimation of *Ureaplasma urealyticum* Growth

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ATP content obtained by luciferin-luciferase luminometry with commercially available reagents provided rapid estimates of *Ureaplasma urealyticum* populations. Each cell contained about 4.7×10^{-18} mol of ATP. We could detect 10^4 CCU_{50} (color change $unit_{50}$) per 100 μl. We correlated urease activity with growth and confirmed the differential response of ureaplasma strains to Mn^{2+}.

Luciferin-luciferase luminometry for the determination of ATP measures the bioluminescence resulting from the specific reaction of the enzyme with its substrates; these include ATP which occurs naturally in the firefly (11, 24). Emitted light is proportional to the amount of ATP present, and, for unicellular organisms, the amount of ATP is roughly proportional to biomass (4) and, thus, should not be affected by cell clumping. ATP values have been used to estimate the number of viable microorganisms (4) as well as to calculate the adenylate energy charge in a sample (1, 2, 8, 9, 21). In diagnostic microbiology, the luciferin-luciferase assay was first used for the detection of significant bacteriuria (5, 24). With the advent of commercially available reagent kits, semiautomated assays have been used to screen for both bacteriuria (10, 13, 15) and bacteremia (3, 14). Luminometry has also been used to determine bacterial susceptibility to antibiotics (6, 12).

In our work with the genital mycoplasma *Ureaplasma urealyticum*, growth detection has presented certain problems. Because of the small size of the organism (ca. 0.5 μm in diameter [18]) and low maximum titers (ca. 10^7 to 10^8 organisms per ml [17]), both microscopic and turbidimetric measurements are unreliable. The highly proteinaceous nondefined media required for the growth of the ureaplasmas preclude the use of a simple spectrophotometric assay. When estimates of cell numbers are needed, viable cell counts are used. CFU determinations on agar underestimate the titers of many strains of ureaplasmas, while the alternative method of measurement, which determines CCU_{50} (color change $unit_{50}$) in broth, gives higher cell counts and a closer correlation between predicted and determined DNA content (23). However, both of these methods require 2 or more days of incubation. Luciferin-luciferase luminometry fulfilled our requirements for immediately available estimates of ureaplasma populations.

The sources of the strains of *U. urealyticum* have been identified (20). Bromothymol blue broth (17) was used for inoculum preparation and for the CCU_{50} method. Preliminary experiments showed that optical quenching in the ATP assay was reduced by eliminating the indicator and by removing medium aggregates by filtration through a membrane of 0.22-μm average pore diameter. This medium, designated B^- broth, was used for cultures to be assayed for ATP. For each test, 100 μl of culture was added to 100 μl of a chemical nucleotide-releasing agent (NRB). After 30 s, 100 μl of purified luciferin-luciferase reagent (Lumit-PM) was injected into this mixture by a Biocounter (model 2010). (Both reagents and the instrument were obtained from Lumac/3M B.V., Schaesberg, The Netherlands.) The relative light units (RLU) emitted were integrated over a 300- to 900-nm range for 10 s. Values for ATP in uninoculated, incubated broth ranged from 65 to 250 RLU and were subtracted from the test readings. The reproducibility of the ATP assay for multiple tests of a single sample and for similar samples on repeated occasions is shown in Table 1. The 74-min generation time of the ATP assay was comparable to the 78-min doubling time calculated from CCU_{50} determinations. Estimates of the generation times for the other 13 serotype standard strains were usually 60 to 90 min, but for one strain, generation time was 5 h.

The growth pattern of the type strain, *U. urealyticum* T960 (CX8), for about eight generations is shown in Fig. 1. The slopes of the ascending curves were similar for changes in CCU_{50} values, ATP amounts, and urease activity as measured by a modified Berthelot reaction (26), as well as for pH. CFU determinations on genital mycoplasma agar (17) made in the lag and logarithmic growth phases and at peak growth were, respectively, 71, 74, and 74% of the corresponding CCU_{50} titers, confirming the greater sensitivity of the latter method. Internal standardization, through the addition of ATP standard to test samples, allowed us to calculate the RLU per μg of ATP. In logarithmic growth phase, each CCU_{50} contained 4.7×10^{-18} mol of detectable ATP. This amount was about 20% of the 2.3×10^{-17} mol of ATP per CFU reported for logarithmic-phase units of *Acholeplasma laidlawii* (1) and only 14% of the 6.4×10^{-17} mol of ATP per CFU we calculated from data for *Spiroplasma citri* (21). Cells of these two genera have genomes at least twice the size of those of *Ureaplasma* spp. (16) and may be larger than ureaplasma cells. After the maximum ureaplasma populations was reached (Fig. 1), the number of CCU_{50} and the rate of urease activity fell, while the RLU demonstrated a more precipitous drop. For *U. urealyticum*, urease activity and urea degradation are believed to be closely linked to energy metabolism (22), although the actual mechanism has not been elucidated.

The initial ATP assays for the growth curve (Fig. 1) detected about 10^5 CCU_{50} per ml or 10^4 CCU_{50} per 100-μl sample of culture. To establish the lower limit of sensitivity, we monitored cultures that had been inoculated with fewer cells. The response of strain 27 (standard for serotype 3) was typical. When that culture contained 1.6 times the RLU of

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TABLE 1. Reproducibility of an ATP assay of *U. urealyticum* T960 (CX8) cultivated in B⁻ broth

Subject of assay	No. of tests	Mean reading (± SD)
Logarithmic-phase culture	20[a]	3,827 (174) RLU
Generation time	11[b]	74 (9) min

[a] Replicate assays of a single logarithmic-phase culture.
[b] Each test result was the mean of two assays.

the broth control (80 versus 48 RLU), the mean of four CCU_{50} determinations was 8.7×10^3 per 100 μl of sample. When the culture contained 3.7 times the RLU of the control, the titer was 9.1×10^4 per 100 μl. Thus, the sensitivity of the method was comparable to the detection of 10^4 to 10^5 larger microorganisms per ml by other workers who have used ATP assays (3, 7, 13–15, 21, 24). We attempted to increase the sensitivity of the assay by concentrating 1.0-ml samples. However, by using vacuum-assisted filtration on prewashed nitrocellulose membranes of 0.22-μm average pore diameter, much of the ATP was lost to the filtrate; about 50% of the total ATP in mid-logarithmic-growth-phase samples and 96% of the peak growth were lost. No viable cells could be detected in the filtrate. These findings, with the data shown in Fig. 1, indicate that the ureaplasma cells were fragile and may have lost structural integrity during the positive phases of growth.

Recently we reported that the serotype standard strains of *U. urealyticum* could be placed in two biotypes on the basis of their differential response to Mn^{2+} (19) as detected by a broth dilution method (25). We reexamined the effect of Mn^{2+} by luminometry. For each of the 14 strains, we used the following equation to compare the slope of growth in broth containing 0.25 mM $MnSO_4$ with that of logarithmic-phase growth in companion cultures without $MnSO_4$: % inhibition = {[(slope in B^-) − (slope in B^- + $MnSO_4$)]/(slope in B^-)} × 100%. Our previous grouping of the 13 strains was upheld. Those we had classified as biotype 1 showed less than 85% inhibition, while those in the biotype 2 group showed more than 85% inhibition. Furthermore, strain 13, which had consistently given an intermediate response in our earlier work and which, as a consequence, had been omitted from the initial scheme, exhibited 73% inhibition and therefore clearly belonged to the biotype 1 cluster with serotype standards 1, 3, 6, and 14.

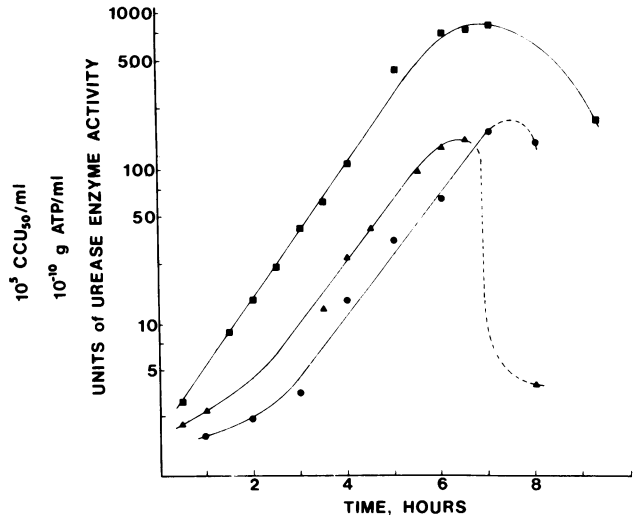

FIG. 1. The growth of *U. urealyticum* T960 (CX8) in B^- broth. ■, CCU_{50} per ml; ▲, RLU of the ATP assay; ●, urease activity. All values were the means of duplicate determinations. The pH of the inoculated culture was 6.2. pH increased to 6.3, 6.4, 6.6, 6.9, 7.1, and 7.2 at 3, 4.5, 5, 6, 7, and 8 h of incubation, respectively.

In addition to these experiments, we have used the ATP assay to determine the MICs of antibiotics for *U. urealyticum* and to evaluate different lots of medium components to be used for its cultivation. Ureaplasmas are among the smallest free-living cells known; cultures produce extremely small cell yields. The successful application of the luciferin-luciferase assay to this species attests to the potential usefulness of the technique for studying the many other fastidious microorganisms which require special consideration in clinical and research laboratories.

Initial experiments were performed by M. Bater. M. Chen provided the photograph.

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