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ATP BIOLUMINESCENCE ASSAY FOR RAPID ANTIBACTERIAL SUSCEPTIBILITY TESTING

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We studied adenosine triphosphate (ATP) synthesized by bacteria in tube dilution cultures to devise a rapid antibacterial susceptibility test. The ATP level was determined with a firefly (*Photinus pyralis*) luciferin/luciferase bioluminescence assay, which allows a direct quantifying of bacteria above 10,000 CFU/ml. At sub-MIC, total bacterial ATP levels were paralleled by increased viable bacterial counts. At above MIC, however, total bacterial ATP levels remained almost constant or decreased gradually. Large differences in total ATP level were observed between sub- and above-MIC levels in the overnight cultures. These differences were also observed in cultures at 6 h. This method may be utilized for rapid antibiotic susceptibility testing.

Key words : Bioluminescence, ATP, Antibacterial susceptibility testing

In antibacterial susceptibility testing, the disc diffusion and tube dilution methods are currently used. These methods are, however, very time-consuming. Hence, requests for rapid results have urged development of quicker susceptibility tests, which include photoelectric measurement of bacterial growth by light absorption or scattering, and chemical or radiometric measurement of some components or metabolites of bacteria after a short incubation time.

ATP is an essential metabolite of all living organisms and its measurement is therefore a good parameter of biomass and cell viability. ATP can be estimated sensitively and simply with a firefly (*Photinus pyralis*) luciferin/luciferase bioluminescence assay[1]. Several attempts have been made to utilize this assay for the detection of bacteria and recently it has become commercially available for screening bacteriuria[2~5]. We examined the suitability of this assay as a rapid antibacterial susceptibility test.

Staphylococcus aureus FDA 209 P and *Escherichia coli* NIHJ JC-2 were used for this study. The susceptibily of these strains to antibiotics was determined as minimal inhibitory concentrations (MICs) with a tube dilution method under the following conditions: tubes containing 10 ml Mueller Hinton broth (MHB) were inoculated with 0.1 ml of the overnight bacterial suspension. After inoculation, the tubes were pre-incubated at 37℃ for one hour. The following antibiotics were tested: ampicillin (ABPC), cefaclor (CCL), fosfomycin (FOM), gentamicin (GM), chloramphenicol (CP), tetracycline (TC), minocycline (MINO) and norfloxacin (NFLX). The antibiotics were dissolved to obtain a concentration of 2,500 μg/ml with appropriate solvents (water, 0.1 M phosphate buffer or 1 N NaOH) and sequential 8-fold dilutions, 313, 39, 5.0, 0.63 μg/ml were prepared with MHB. Then 0.4, 0.2 and 0.1 ml of these stock solutions were dispensed to the pre-incubated tubes to obtain a two-fold series of final concentrations, from 100 to 0.006 μg/ml. After 24 h of incubation at 37℃, the MICs were determined as the lowest concentration at which there was no turbidity on visual inspection.

ATP levels synthesized by bacteria were deter-

mined using the following procedure: inoculation of bacteria, pre-incubation and dispensation of antibiotics were carried out under the conditions described above. Tubes without drug served as growth controls. The test specimens were obtained at intervals during incubation at 37℃. One millilitre of each specimen was mixed with 10 ml of 0.025 M HEPES buffer (pH 7.75). The mixture was centrifuged for 10 min at 3,000 × g at 4℃. The supernate was washed away and subsequently 1 ml of the same buffer was added to the precipitate. A 0.1 ml ATP-releasing reagent (Labo Science, Tokyo, Japan) was pipetted into the tube to extract intra-bacterial ATP. After 1 min at room temperature, a 0.1 ml aliquot of the mixture was pipetted into a cuvette placed in the counting chamber of the Lumi-photometer TD-4000 (Labo Science, Tokyo, Japan). The measurement of bioluminescence was started by adding 0.1 ml of luciferin-luciferase reagent (ATP bioluminescence HS, Boehringer Mannheim Biochemica, FRG) into the cuvette. The light emission was measured after a 5-sec delay and the luminescence parallel to bacterial ATP was expressed as relative light units (RLUs) accumulated over a 15-sec integration. The ATP level in the specimen was calculated by using the standard curve of ATP as a reference after correcting for background light emission. Numbers of viable bacterial cells were counted as CFU/ml with an agar-plating method after serial dilution with saline.

The ATP content corresponding to 1 CFU of bacterium was calculated as approximately 2×10^{-18} mol for *S. aureus* FDA 209 P and 1×10^{-18} mol for *E. coli* NIHJ JC-2. With this bioluminescence assay, ATP concentration can be measured in a range from ten picomoles in a final volume of 0.1 ml. Hence, 10,000 CFU/ml of bacteria are directly detectable[5].

Bacterial ATP levels synthesized and accumulated in a drug-free control culture were paralleled to increase the viable cell counts (Fig. 1). The variations in the bacterial ATP levels corresponding to 1 CFU of bacterium rarely exceeded one logarithmic order despite phases of bacterial growth.

Fig. 2 shows the time course of viable cell counts and bacterial ATP levels in *E. coli* NIHJ JC-2

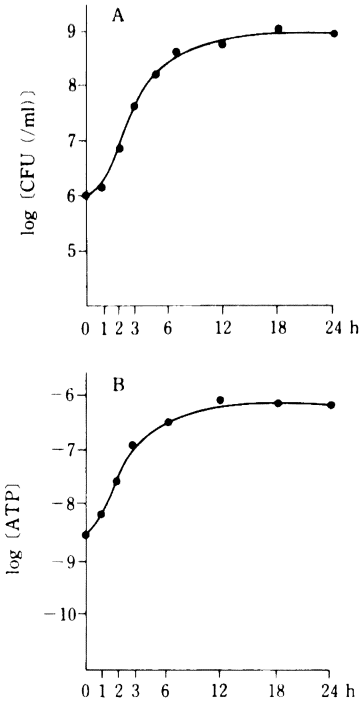

Fig. 1. Growth of *Escherichia coli* NIHJ JC-2 monitored by
A: viable cell counts.
B: bacterial ATP level.

culture exposed to sub- and above-MIC of CCL and MINO. Bacterial ATP levels exposed to sub-MIC increased almost proportional to the viable cell counts, which were similar to those of drug-free control cultures. On the other hand, those exposed to above-MIC held constant or decreased gradually after adding these drugs. (Note that bacterial ATP levels did not correlate with viable cell counts under these conditions.)

Fig. 3 shows bacterial ATP levels in overnight culture exposed to drugs diluted serially. With increasing concentration of drugs, bacterial ATP levels decreased. Also large differences in ATP levels were observed around the MIC, its value almost corresonding to the concentration where a steep downward curve was obtained. Large differences in ATP levels around the MIC were also observed 6 h after adding the drug (Fig. 4).

The ATP bioluminescence assay is technically

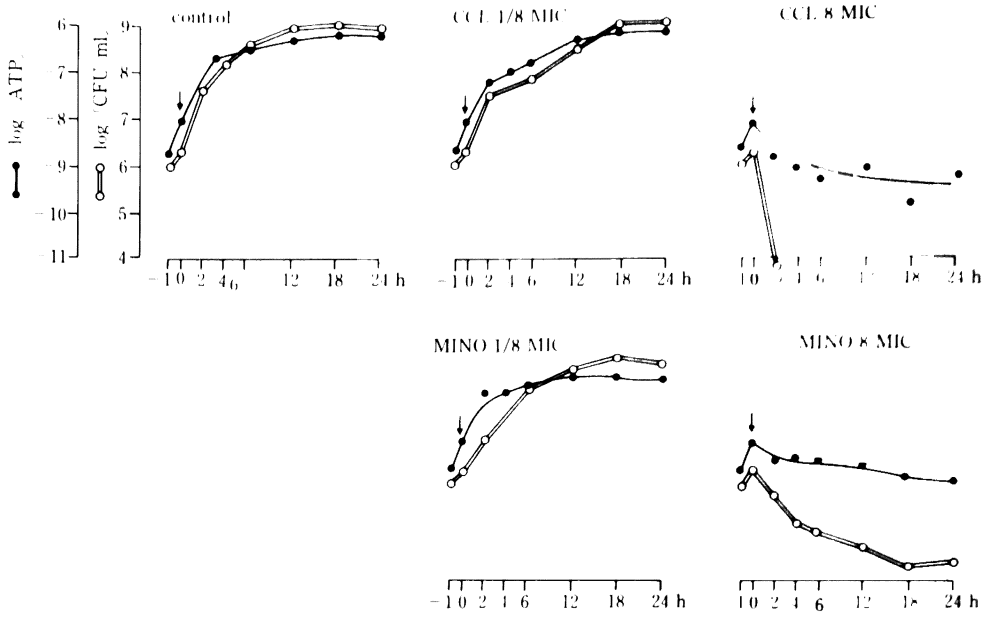

Fig. 2. Relation between viable cell counts (○) and bacterial ATP level (●) in culture of *Escherichia coli* NIHJ JC-2 exposed to cefaclor (CCL) and minocycline (MINO)

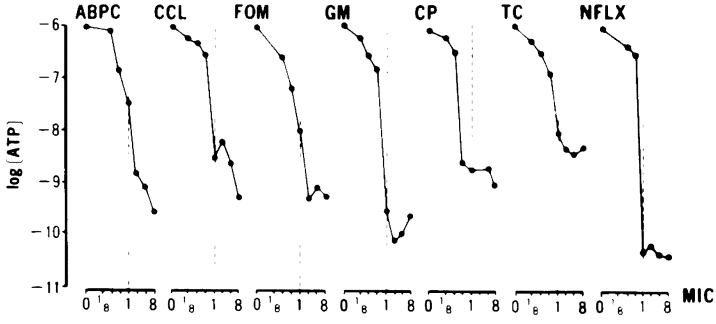

Fig. 3. Bacterial ATP level in overnight culture of *Escherichia coli* NIHJ JC-2 exposed to drugs diluted serially

ampicillin (ABPC), cefaclor (CCL), fosfomycin (FOM), gentamicin (GM), chloramphenicol (CP), tetracycline (TC), norfloxacin (NFLX)

simple and highly sensitive. It is capable of direct evaluation of bacterial concentration, which has made it an attractive alternative to classical microbiological methods by facilitating overnight culture[1] In principle, it should be possible to use bacterial ATP measurement to assess antibiotic-induced growth inhibition as an alternative to, for example, photoelectric measurements[6,7]

At sub-MIC, viable cell counts and ATP levels synthesized and accumulated by bacteria increassed in parallel. In contrast at above-MIC, the line of bacterial ATP held constant and diverged from that of viable cell counts. In general, addition of bacteriostatic antibiotics caused relatively slow regression in viable cell counts and constant ATP levels were maintained. On the other hand, addition of bacter-

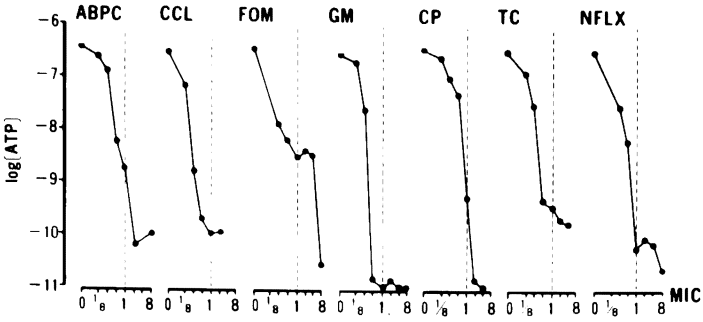

Fig. 4. Bacterial ATP level of *Escherichia coli* NIHJ JC-2 at 6 h after dispensation of antibacterial drugs

iocidal antibiotics caused rapid cell lysis (as indicated by phase-contrast microscopic observation) or rapid decrease in viable cell counts, though considerable smounts of ATP still existed. Although this phenomenon is surprising electron microscopic observation suggests that the discrepancy presumably stems from an accumulation of ATP from bacterial debris with minimal cell viability.

Despite the ATP accumulation observed in above -MIC tubes, much higher levels of ATP were synthesized in overnight-cultured sub-MIC tubes. The higher the concentration of antibiotics added, the less were the amounts of accumulated ATP. The steep downward curve of ATP level exposed to higher concentrations of antibiotics showed good agreement in parallel MIC tests with a tube dilution method. These results were also observed in culture at 6 h. In detail, there were minor discrepancies between the MIC endpoints and the concentrations where such downward curves were observed. But these are thought to be sufficiently negligible from a practical point of view, and do not affect the utility of this assay for rapid antibiotic susceptibility.

Studies must be pursued further, however, to include several bacteria, especially fastidious strains and antibiotics with different modes of action, and to estimate the clinical relevance of this assay.

In conclusion, with the bioluminescence assay, the effects of antibiotics on the synthesis of bacterial ATP are rapidly observable, which may render this a useful technique for rapid antibiotic susceptibility testing.

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ATP生物発光法による迅速な抗菌剤感受性試験

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細菌の抗菌剤感受性を，液体培地中で増殖した細菌のATPの総量を測定することにより，迅速に判断する方法について検討した。ATPの定量は生物発光法により行ったが，本法によれば，おおむね10^4，CFU/ml以上の細菌数の即時定量が可能であった。MIC未満の抗菌剤濃度では，ATP総量は生菌数の増加と平行して増加した。MIC以上では，ATP総量は不変または軽度減少を示した。液体希釈法で昼夜培養を行った場合，MICの前後でATP総量に大きな差異が認められたが，この現象は6時間程度の短時間培養でも観察され，ATP定量による迅速抗菌剤感受性の可能性が示唆された。

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