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Adenylate Energy Charge in *Acholeplasma laidlawii*

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Adenosine 5'-triphosphate, adenosine 5'-diphosphate, and adenosine 5'-monophosphate were produced by *Acholeplasma laidlawii* B-PG9 growing in modified Edward medium. The adenylate energy charge was calculated to be 0.84 ± 0.07 and ranged from 0.91 to 0.78 during exponential growth (12 to 24 h). During exponential growth, *A. laidlawii* contained, at 17.5 h, 2.3×10^{-17} mol of adenosine 5'-triphosphate per colony-forming unit and, at 16 h, 27.3 nmol of adenosine 5'-triphosphate per mg (dry weight). The medium supported a doubling time of 0.95 h. The molar growth yields ($Y_{glucose}$ = grams [dry weight] per mole of glucose used) were 40.2 ± 3.4 (16 h) and 57.1 ± 9.7 (20 h) during midexponential growth. A maximum yield of 8.3×10^9 colony-forming units was reached at 24 h, when 56% of the initial concentration of glucose had been used. At 40 h, during the stationary phase, 14.95 ± 3.75 μmol of glucose per ml of medium had been used. At this time, the culture fluids contained 21.86 ± 0.37 μmol of lactate per ml and 3.14 ± 0.13 μmol of pyruvate per ml.

Energy production in *Mollicutes* strains is not well understood. Most, if not all, ATP is presumed to be synthesized at the substrate level because these microorganisms lack quinones and cytochromes (8, 16). Enzymatic activity at sites understood to be capable of substrate phosphorylation has been demonstrated in various *Mollicutes* strains; for example, Smith reported the presence of glutamine synthetase in nonfermentative strains of *Mycoplasma* (22). The arginine dihydrolase pathway was shown to be present in nonfermentative isolates by Smith (21) and by Schimke and Barile (20). Kahane et al. demonstrated acetate kinase activity in cytoplasmic fractions of *Acholeplasma laidlawii* and *Mycoplasma hominis* (9). Romano et al. have indicated that a reversal of the adenosine triphosphatase in *Ureaplasma urealyticum* can occur and suggest this as a possible mechanism for ATP production in this organism (18).

The production of the adenine nucleotides ATP, ADP, and AMP in growing *Mollicutes* strains has been detected only in *Spiroplasma citri* by Saglio et al. (19). Tarshis et al. did not find ATP in *A. laidlawii* strain Koller (23). We chose to quantitate cellular adenylates and the medium content of pyruvate and lactate during growth of *A. laidlawii* B-PG9 to study energy production and fermentative pathways in a representative *Mollicutes* strain. The changing adenylate interrelationships were expressed as the adenylate energy charge (EC_A), which equals ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]) (1, 10).

MATERIALS AND METHODS

Culture and medium. *A. laidlawii* B-PG9 was grown in our modification of a recipe for Edward medium (17). The basal medium contained 21 g of PPLO broth (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract (Difco), 100 mg of herring calcium-DNA (Mann Research Laboratories, New York, N.Y.), 11 g of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Calbiochem, San Diego, Calif.), and 1 liter of distilled water. The basal medium was adjusted to pH 7.5 with NaOH and autoclaved. The following additions were made per liter of sterile basal medium: 14 ml of 50% (wt/vol) aqueous sterile (by autoclaving) glucose; 6 ml of 28% (wt/vol) aqueous sterile (by autoclaving) K_2HPO_4; 100,000 U of penicillin G; and 5.0 ml of horse serum (control lot 268095 or 200011H; KC Biologicals, Lenexa, Kans.), which we heat reinactivated (56°C, 1 h).

Cultivation conditions. A 300-ml portion of a 24-h starter culture was inoculated into 3 liters of temperature-equilibrated culture medium in a 6-liter flask. Cultures were incubated statically at 37°C.

Assays. Samples of culture fluids were assayed after 12, 16, 20, 24, and 40 h of incubation for pH, turbidity, and viable counts (colony-forming units [CFU]) (3). After centrifugation (15,000 × *g*, 15 min), samples of cell-free supernatant were frozen and later examined for lactate, pyruvate, and glucose content. Washed cell pellets (15) were assayed for protein by the procedure of Lowry et al. (12).

Samples (1 liter each) of culture fluid were centrifuged at 15,000 × *g* for 15 min. Less than 5 s after centrifugation, the pellet was suspended in 25 ml of boiling 5 mM Tris buffer (pH 7.8) (10, 19). The hot suspension was placed for 10 min in a boiling water bath and then centrifuged at 27,000 × *g* for 15 min. The supernatant was removed and filtered through a

Pasteur pipette with a small plug of glass wool. The extracted pellet was washed three times with 10 ml of buffer. The washes were also filtered through the glass wool-plugged column. The filtrates were pooled and refiltered through glass wool. The pooled filtrate was frozen at −70°C, lyophilized, and stored in vacuo over phosphorus pentoxide at −40°C.

ATP was measured with the hexokinase, glucose-6-phosphate dehydrogenase procedure described by Bostick and Asmus (2). ADP was measured by first converting it to ATP with yeast pyruvate kinase (Mann), as described by Kimmich et al. (11). The ADP concentration was calculated as the difference between the concentrations of ATP in the sample before and after conversion of ADP to ATP (2, 11). The total adenylate concentration was determined by converting all the adenines to adenosines with calf intestinal alkaline phosphatase (EC 3.1.3.1) and then converting the adenosines to inosines with adenosine deaminase (EC 3.5.4.4) (2). AMP was estimated by subtracting the sum of the previously determined ATP and ADP concentrations from the total adenylate concentration (2).

Lactate was measured by conventional procedures with NAD and lactate dehydrogenase (13). Pyruvate was measured through its conversion to lactate with lactate dehydrogenase, as described by Czok and Lamprecht (7). All lactate and pyruvate values were corrected for the small amounts of these substances in the uninoculated medium. Residual glucose was determined with the glucose oxidase-peroxidase assay (Glucostat; Worthington Biochemicals Corp., Freehold, N.J.) after $Ba(OH)_2$-$ZnSO_4$ deproteinization (26).

Reagents. All biochemicals and enzymes were purchased from Sigma Chemical Company (St. Louis, Mo.) unless otherwise specified. All spectrophotometry was performed in a Gilford 240 (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) or Beckman DB (Beckman Instruments, Inc., Fullerton, Calif.) spectrometer.

Statistical analyses. All data presented in the figures are the means of five experiments and, except for the logarithmically expressed CFU data, the corresponding *n*th order regression analysis is presented as the coefficient of correlation, the standard error of the estimate, and the regression order for each data set. These analyses were performed on an Apple II Computer (Apple Computers Inc., Cupertino, Calif.) programmed by M. Petricevic (Scott Research Laboratories, Cleveland, Ohio). Averaged data in the text are followed by their standard deviations.

RESULTS

Growth characteristics. The data for CFU and turbidity are shown in Fig. 1A. The maximum yield of *A. laidlawii*, 8.3 × 10^9 CFU/ml, was reached at approximately 24 h. The growth rate was maximal between 16 and 20 h of incubation, as monitored by both turbidity and CFU. The doubling time of the organism during this period was 0.95 h.

The pH of the medium decreased from 7.58 ± 0.06 to 6.01 ± 0.29 (n = 5) (Fig. 1B). This decrease was experimentally duplicated by the addition of 38.6 ± 3.9 mmol of HCl per liter of uninoculated medium (n = 5). After 40 h of incubation, culture fluids contained 21.86 ± 0.37 mmol of lactate per liter (n = 4) and 3.14 ± 0.13 mmol of pyruvate per liter (n = 4).

Changes in the glucose, lactate, and pyruvate concentrations in the medium during growth are shown in Fig. 1B and were compared with the CFU data in Fig. 1A. The pyruvate concentration increased during the exponential growth phase. The lactate concentration increased through the exponential and stationary growth

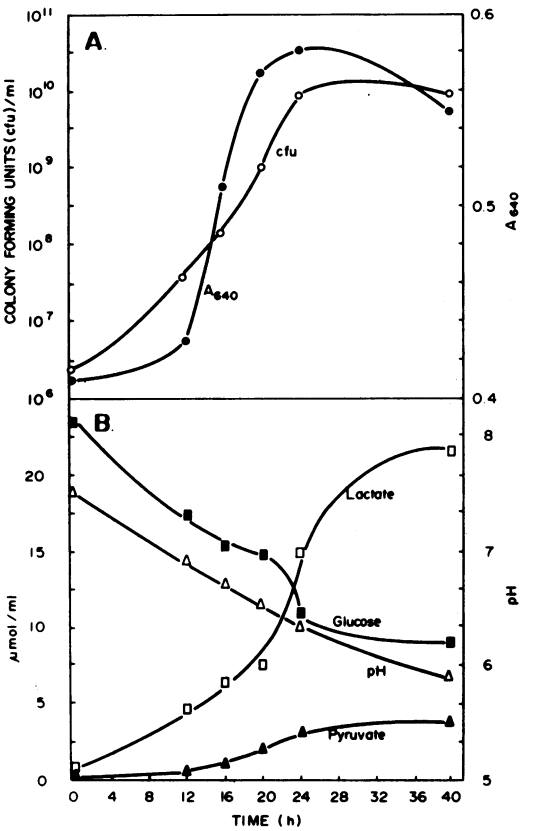

FIG. 1. *Growth characteristics of A. laidlawii B-PG9 in modified Edward medium. At the indicated times, samples were removed and analyzed as described in the text. The results are plotted, except for the logarithmically presented CFU, as the best fit nth order regression line. (A) CFU and turbidity at an absorbance at 640 nm (A_{640}) (coefficient of correlation, 0.99; standard error of the estimate, 0.02; and regression order, 2nd). (B) Medium components. The coefficient of correlation, standard error of the estimate, and regression order values were, respectively, 0.98, 1.42, and 2nd for residual glucose, 0.97, 2.25, and 2nd for lactate, 0.91, 0.69, and 2nd for pyruvate, and 0.99, 0.07, and 2nd for pH.*

phases. The molar growth yields ($Y_{glucose}$ = grams [dry weight] per mole of glucose used) were calculated to be 40.2 ± 3.4 at 16 h (n = 4) and 57.1 ± 9.7 at 20 h (n = 5).

Adenylate production. ATP, ADP, and AMP were produced by *A. laidlawii* B-PG9. The average changes in cellular EC_A, ATP, ADP, AMP, and total adenylates during growth are shown in Fig. 2A and 2B. These data were compared with the CFU data in Fig. 1A. The ATP content per milliliter of culture increased during the exponential growth phase and decreased during the stationary growth phase. The ADP concentration was relatively low throughout all phases of growth. The AMP concentration increased during the stationary growth phase. The total adenylate concentration generally paralleled the ATP concentration.

The mean EC_A between 12 and 24 h of growth was calculated to be 0.84 ± 0.07 (range, 0.91 to 0.78; n = 12). The mean cellular ATP concentration (10^{-17} mol of ATP per CFU) was found to decrease during growth (11.8, 3.96, 1.69, 0.20, and 0.098 at 12, 16, 20, 24, and 40 h, respectively).

The mean cellular ATP concentrations (nanomoles of ATP per milligram [dry weight]) (n = 5) were 27.3 ± 0.23 and 27.6 ± 0.57 at 16 and 20 h, respectively. The mean cellular total adenylate concentrations (nanomoles of total adenylate per milligram [dry weight]) (n = 5) were 53.7 ± 4.2 and 34.6 ± 4.1 at 16 and 20 h, respectively.

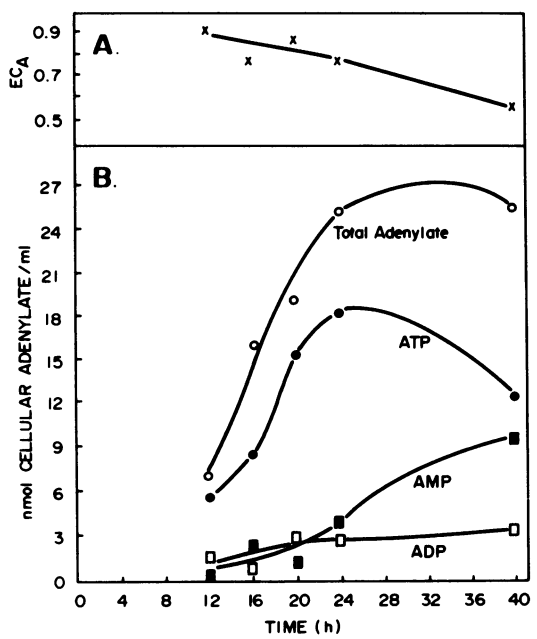

FIG. 2. *Cellular EC_A and adenylate content in A. laidlawii B-PG9 grown in modified Edward medium. At the indicated times, samples were removed and analyzed as described in the text. The results are plotted as the best fit nth order regression line. (A) EC_A (coefficient of correlation, 0.94; standard error of the estimate, 0.07; and regression order, 2nd). (B) Adenylate content. The coefficient of correlation, standard error of the estimate, and regression order were, respectively, 0.99, 2.45, and 3rd for total adenylate, 0.99, 1.52, and 3rd for ATP, 0.87, 1.06, and 3rd for ADP, and 0.96, 2.06, and 3rd for AMP.*

DISCUSSION

The doubling time of 0.95 h was comparable to the longer time of 1.30 h reported for *A. laidlawii* A-PG8 in Edward medium (3).

We found, at 24 h (late exponential growth phase), with 8.3 × 10^9 CFU/ml, that there was ca. 13 μmol of glucose per ml of culture present or ca. 56% of the starting glucose concentration. At 40 h, the medium contained 1.46 mol of lactic acid per mol of glucose utilized. Neimark and Pickett reported the accumulation of 1.07 mol of lactate per mol of glucose utilized by *A. laidlawii* A (14). At 40 h, the medium contained 0.21 mol of pyruvate per mol of glucose utilized. Accumulation of pyruvate in cultures was also reported by Tourtellotte and Jacobs, who suggested that the increase was due to insufficient α-lipoic acid (24).

We speculate that *A. laidlawii* B-PG9 may be capable of succinate synthesis by way of phosphoenolpyruvate, oxaloacetate, malate, and fumarate, a view supported, in our opinion, by the presence of fumarase and malate dehydrogenase in *Mycoplasma arthritidis* (25) and by CO_2 fixation by *A. laidlawii* indicated in both our preliminary experiments and suggested by an earlier report (4).

Chapman and Atkinson believe that the frequently reported EC_A value of ca. 0.8 is slightly low and that in most, if not all, normally metabolizing bacterial cells the value is near 0.9 (5). EC_A values below 0.8 are attributed to the degradation of ATP resulting from slow handling procedures, prolonged centrifugation, filtration, and washing (5). *Mollicutes* strains are damaged by washing and filtration, and in our experience *A. laidlawii*, grown in a relatively low serum concentration, has been the most fragile *Mollicutes* organism (15). We elected to use our reported procedure with reservation, as the elapsed time from harvest to extraction was 15 to 20 min, and times less than 1 min are prescribed. Nevertheless, the data suggest that our procedures were adequate because, during exponential growth, the mean EC_A was 0.84 ± 0.07 (range, 0.91 to 0.78; n = 12), a value within the limits suggested by Chapman and Atkinson (5)

and higher than that reported for many other microbes (6, 10). As we expected, the EC_A varied with the portion of the growth curve, remaining high and relatively constant during the exponential growth phase and decreasing during the stationary growth phase. Further, our results are compatible with the data of Saglio et al., who reported an EC_A of ca. 0.85 during exponential growth of the *Mollicutes* organism *S. citri* strain Israeli (NCPPB 2565) (19). Based on their work we computed a value of 1.9×10^{-17} mol of ATP per CFU for *S. citri* during midexponential growth (24 h). We found, during midexponential growth (17.5 h) of *A. laidlawii* B-PG9, a value of 2.3×10^{-17} mol of ATP per CFU or 1.39×10^{7} molecules of ATP per CFU. The mean cellular ATP concentration, reported as nanomoles of ATP per milligram (dry weight), was higher than that reported for other microorganisms, and this disparity may reflect the absence of a cell wall, which contributes considerably to the dry weight of walled microorganisms (10).

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