Synthesis of Adenylate Nucleotides by Mollicutes (Mycoplasmas)

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Cultures of the Mollicutes (mycoplasmas) Acholeplasma laidlawii B, Acholeplasma morum, Mycoplasma bovis, Mycoplasma arginini, Mycoplasma fermentans and Mycoplasma gallisepticum, representing four metabolic groups, were sampled at intervals over a 40 to 50 h period and assayed for the numbers of c.f.u., changes in pH and glucose concentration, and concentrations of ATP, ADP, AMP, lactate and pyruvate. The adenylate energy charge (ECA), the mean generation time, and the number of nmol of ATP (mg dry weight)^{-1} were calculated for cultures in the mid-exponential growth phase. The maximum cell concentrations ranged from 0.2 \times 10^{10} to 5.0 \times 10^{10} c.f.u. ml^{-1}. Doubling times ranged from 0.34 to 3.29 h. The fermentative, non-arginine-requiring A. laidlawii B, A. morum, and M. gallisepticum, as well as the fermentative, arginine-requiring M. fermentans, utilized glucose and produced lactate and pyruvate. The non-fermentative, non-arginine-requiring M. bovis neither utilized glucose nor produced lactate or pyruvate. The non-fermentative, arginine-requiring M. arginini utilized glucose, but did not produce lactate or pyruvate. At mid-exponential growth phase, the average ECA of A. laidlawii B was 0.90, a value similar to that reported for Spiroplasma citri and other bacteria. In contrast, the average ECA of A. morum and the four Mycoplasma species was 0.70. In A. laidlawii B at mid-exponential growth phase, ATP accounted for 97\% of the total adenylate nucleotide pool. At the same stage of growth, the average cellular ATP concentration of the other Mollicutes was significantly lower, ranging from 45 to 63\% (P < 0.01). Excluding A. laidlawii B, the Mollicutes were relatively energy deficient during their mid-exponential growth phase. The diminished metabolic capacity may be related to the association of Mollicutes with living cells and perhaps to the cytopathic effects of these micro-organisms.

INTRODUCTION

Some wall-less, facultatively anaerobic Mollicutes (mycoplasmas) produce ATP to cellular concentrations of about 4 mm (Rottem et al., 1981) or about 2 \times 10^{-17} mol ATP per c.f.u. (Beaman & Pollack, 1981) and attain an adenylate energy charge (ECA) value of 0.85 (Saglio et al., 1979; Beaman & Pollack, 1981). This ECA value is characteristic of normally metabolizing cells (Chapman & Atkinson, 1977). As Mollicutes apparently lack quinones and cytochromes (Hollaender et al., 1977; Pollack, 1975; Pollack et al., 1981) their production and use of ATP must proceed by mechanisms which compensate for the absence of parts or all of the electron transport system. Our earlier studies (Beaman & Pollack, 1981) indicated that the ECA value of the most frequently studied non-fastidious saprophyte, the fermentative, non-arginine-requiring Acholeplasma laidlawii B-PG9 during exponential growth was compatible with normally metabolizing cells, and this organism may have more ATP (mg dry wt)^{-1} than many other micro-organisms. As part of a continuing study designed to demonstrate the loci where, and the pathways by which, Mollicutes synthesize ATP, it was necessary to determine their cellular concentration of adenylate nucleotides during growth. We studied representative species of Mollicutes selected from the four prominent metabolic groups: these were the fermentative and non-fermentative groups, and within each, the arginine or non-arginine-requiring species.
Examination of these species of Mollicutes revealed that they had significantly lower ECA values at their mid-exponential growth phase than *A. laidlawii* B. Furthermore, the mean ECA value and ATP concentration of all Mollicutes, except for *A. laidlawii* B, were lower than those generally reported for other microbes (Chapman & Atkinson, 1977; Karl, 1980).

**METHODS**

**Organisms.** Six Mollicutes representing the four generally recognized metabolic groups were selected from our stock collection. These were the three fermentative, non-arginine-requiring *Acholeplasma laidlawii* B-PG9, *Acholeplasma morum* S2, and *Mycoplasma gallisepticum* S6; the fermentative, arginine-requiring *Mycoplasma fermentans* PG18; the non-fermentative, non-arginine-requiring *Mycoplasma bovis* PG45; and the non-fermentative, arginine-requiring *Mycoplasma arginini* G230. *Escherichia coli* ATCC 29522 was also examined.

**Medium and cultivation.** All organisms were grown in modified Edward medium (Beam & Pollack, 1981). The medium was supplemented with heat-inactivated horse serum (2%, v/v; lot no. 2001H, K.C. Biologicals, Lenexa, Kan., U.S.A.) for the growth of the two *Acholeplasma* species; 5% (v/v) serum was added for the growth of the four *Mycoplasma* species. The medium was additionally supplemented with 0.1% (w/v) L-arginine. HCl for the growth of *M. fermentans* and *M. arginini*. *Escherichia coli* was grown without horse serum and without added arginine.

Media at 37 °C were inoculated with 10% (v/v) of starter cultures growing in the same medium. All cultures were incubated without shaking at 37 °C.

**Assays of culture fluids.** Samples of culture fluids were taken at various times during growth phases and assayed for pH, turbidity, and viable counts (c.f.u.) (Butler & Knight, 1960). After centrifugation (15000 g at 4 °C for 15 min; GSA rotor, Sorvall) samples of cell-free supernatant were frozen and subsequently concentrations of lactate, pyruvate, and glucose were determined. Cells were washed as described earlier (Pollack, 1975). Washed cells were assayed for protein by the Lowry procedure or lyophilized for dry weight determinations.

**Extraction and measurement of cellular adenylate nucleotides.** Adenylate nucleotides were extracted by our modification of the method of Robrish *et al.* (1978). A 0.2 ml sample of the culture was added to 1.8 ml 5 mM-Tris buffer, pH 7.5, at 95 °C in a test tube (13 × 100 mm) and immediately placed in a 110 °C heat block for 2 min. The sample was capped with a marble. The tubes were transferred to an ice-bath, then frozen and stored at −20 °C until assayed.

ATP was assayed by the luciferin–luciferase method (Kimmich *et al.*, 1975). Light emission was measured in a Beckman LS 7000 liquid scintillation spectrometer. The coincidence circuitry was disconnected for the assay.

Firefly extract was prepared following the procedure of Kimmich *et al.* (1975). Commercial preparations of firefly tails were suspended in assay buffer containing 20 mM-glycylglycine (pH 7.8), 5 mM-sodium arsenate and 4 mM-magnesium sulphate. Calcium phosphate was added to remove interfering substances and the mixture was placed on ice for 10 min with intermittent shaking. The precipitate was removed by centrifugation and the supernatant (firefly extract) was removed and kept on ice until used, within one week.

The sample to be assayed (50 µl) was added to 1-9 ml assay buffer in a vial already in place in the liquid scintillation spectrometer at 19 to 22 °C. At timed intervals, 50 µl firefly extract was added to the reaction mixture which was immediately assayed for ATP. ADP and AMP were assayed after conversion to ATP by using pyruvate kinase or myokinase and pyruvate kinase, respectively (Kimmich *et al.*, 1975). Concentrations of ADP and AMP were calculated as the difference in concentrations of ATP before and after enzymic conversion of ADP and AMP to ATP (Bostick & Asmus, 1978). We detected no ATP, ADP or AMP in our uninoculated media. We were unable to detect less than 750 pmol of any adenylate nucleotide (ml medium)−1. Total adenylate nucleotide values are the sum of the ATP, ADP and AMP concentrations.

The adenylate energy charge (ECA) was calculated according to Chapman & Atkinson (1977) as:

\[
EC_A = \frac{[\text{ATP}]}{[\text{ADP}]} + 0.5 \frac{[\text{ADP}]}{[\text{ATP}]} + [\text{ADP}] + [\text{AMP}].
\]

**Chemical assays.** Lactate was measured by conventional procedures with NAD and lactate dehydrogenase (Lundholm *et al.*, 1963). Pyruvate was measured through its conversion to lactate with lactate dehydrogenase, as described by Czok & Lamprecht (1974). 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, Freehold, N.J. U.S.A.) after Ba(OH)_2-ZnSO_4 deproteinization (Washka & Rice, 1961). We are unable to detect less than 150 nmol glucose, 1.0 nmol lactic acid, or 1.5 nmol pyruvic acid (ml medium)−1.

**Reagents.** All biochemicals and enzymes were purchased from Sigma unless otherwise specified. All spectrophotometry was performed in a Gilford 240 or Beckman DB spectrometer.

**Statistical analyses.** Curves presented in the Figures were statistically derived from the raw data of three to seven separate experiments. Except for the logarithmically expressed c.f.u. data, the nth order regression analysis is indicated in the respective Figure legend along with the coefficient of correlation, the s.e. of the estimate, and
ATP, ADP and AMP from growing Mollicutes

Fig. 1. Adenylate nucleotide content, ECₐ, and growth characteristics of A. laidlawii B-PG9. At indicated times, samples were removed and analysed as described in the text. The accumulated results from five separate batches of cells are plotted, except for the logarithmically presented c.f.u. (○), as the best fit nth order regression line. The coefficient of correlation, s.e. of the estimate, and regression order were, respectively: 0.99, ± 0.24 nmol and 3rd for total adenylate nucleotides (△); 0.98, ± 1.29 nmol and 3rd for ATP (■); 0.65, ± 0.42 nmol and 3rd for ADP (◇); 0.93, ± 0.56 nmol and 3rd for AMP (+); 0.94, ± 0.56 and 2nd for ECₐ (●); 0.98, ± 1.42 µmol and 2nd for residual glucose (▼); 0.97, ± 2.25 µmol and 2nd for lactate (×); 0.91, ± 0.69 µmol and 2nd for pyruvate (▲); and 0.99, ± 0.97 and 2nd for pH (□). The bar indicates the extent of exponential phase.

Fig. 2. Adenylate nucleotide content, ECₐ, and growth characteristics of A. morum S2. At indicated times, samples were removed and analysed as described in the text. The accumulated results from three separate batches of cells are plotted, except for the logarithmically presented c.f.u. (○), as the best fit nth order regression line. The coefficient of correlation, s.e. of the estimate and regression order were, respectively: 0.96, ± 1.69 nmol and 3rd for total adenylate nucleotides (△); 0.99, ± 0.14 nmol and 3rd for ATP (■); 0.99, ± 0.19 nmol and 3rd for ADP (◇); 0.96, ± 1.59 nmol and 3rd for AMP (+); 0.96, ± 0.05 and 2nd for ECₐ (●); 0.86, ± 3.5 µmol and 2nd for glucose (▼); 0.99, ± 0.19 µmol and 2nd for lactate (×); 0.97, ± 0.29 µmol and 2nd for pyruvate (▲); and 0.99, ± 0.01 and 2nd for pH (□). The bar indicates the extent of exponential phase.

the regression order for each data set. These analyses were performed on an Apple II computer (Apple Computers, Cupertino, Calif., U.S.A.) programmed by M. Petricevic (Scott Research Laboratories, Cleveland, Ohio, U.S.A.). In the text, mean values are followed by their s.d.

RESULTS

Growth characteristics

The multiplication of Mollicutes is reported as the number of c.f.u., and the collected data are shown in Figs 1 to 6. The maximum number of c.f.u. for each strain ranged from 4.5 × 10¹⁰ ml⁻¹ at 28 h for M. gallisepticum to 2.3 × 10⁹ ml⁻¹ at 40 h for A. morum (Figs 1 to 6). The shortest mean generation time was 0.34 h for A. laidlawii and the longest was 3.29 h for M. arginini.

The fermentative organisms utilized glucose and produced lactate (Figs 1 to 4). Pyruvate was produced by all fermentative organisms except M. fermentans (Fig. 4). The pH of the cultures of the fermentative organisms also decreased during growth (Figs 1 to 4). Most acid was produced by A. laidlawii: in three experiments the pH of the medium decreased from 7.58 ± 0.06 to 6.01 ±
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Fig. 3. Adenylate nucleotide content, ECₐ, and growth characteristics of M. gallisepticum. At indicated times, samples were removed and analysed as described in the text. The accumulated results from seven separate batches of cells are plotted, except for the logarithmically presented c.f.u. (○), as the best fit nth order regression line. The coefficient of correlation, s.e. of the estimate, and regression order were, respectively: 0.93, ±1.35 nmol and 3rd for total adenylate nucleotides (△); 0.89, ±1.41 nmol and 3rd for ATP (■); 0.83, ±1.69 nmol and 3rd for ADP (□); 0.96, ±1.05 nmol and 3rd for AMP (+); 0.72, ±0.52 and 2nd for ECₐ (●); 0.98, ±1.05 μmol and 2nd for glucose (▲); 0.99, ±0.61 μmol and 2nd for lactate (×); 0.80, ±0.29 μmol and 2nd for pyruvate (▲); and 0.99, ±0.15 and 2nd for pH (□). The bar indicates the extent of exponential phase.

Fig. 4. Adenylate nucleotide content, ECₐ, and growth characteristics of M. fermentans PG18. At indicated times, samples were removed and analysed as described in the text. The accumulated results from three separate batches of cells are plotted, except for the logarithmically presented c.f.u. (○), as the best fit nth order regression line. The coefficient of correlation, s.e. of the estimate, and regression order were, respectively: 0.99, ±1.47 nmol and 3rd for total adenylate nucleotides (△); 0.65, ±2.47 nmol and 3rd for ATP (■); 0.99, ±0.19 nmol and 2nd for ADP (□); 0.90, ±0.82 nmol and 3rd for AMP (+); 0.75, ±0.18 and 2nd for ECₐ (●); 0.96, ±3.28 μmol and 2nd for glucose (▲); 0.88, ±1.19 μmol and 2nd for lactate (×); 0.81, ±0.61 μmol and 2nd for pyruvate (▲); and 0.96, ±0.10 and 2nd of pH (□). The bar indicates the extent of exponential phase.

0.29 (Fig. 1). This decrease was experimentally duplicated by the addition of 38.6 ± 3.9 mmol of HCl per litre of uninoculated medium (n = 5). The non-fermentative organism M. arginini also utilized glucose during growth, but M. bovis did not (Figs 5 and 6). Neither organism produced lactate or pyruvate during growth. During the growth of M. arginini the pH of the medium increased from 7.58 ± 0.06 to 7.9 ± 0.39 (n = 3) (Fig. 5); however, the pH of the medium remained unchanged during the growth of M. bovis (Fig. 6).

Adenylate production

ATP, ADP, and AMP were detected in cultures of all Mollicutes examined and the total adenylate nucleotide concentration increased during growth (Figs 1 to 6). A major finding was that in A. laidlawii cultures, during mid-exponential growth phase, ATP accounted for 97% of the total adenylate nucleotide pool, while the mean ATP concentrations in the cultures of the other Mollicutes during the same period were significantly lower (P < 0.01) and accounted for 45 to 63% of the total nucleotide pool (Table 1). The AMP concentration of all Mollicutes tested
ATP, ADP and AMP from growing Mollicutes

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except *A. laidlawii* rose during exponential growth phase (Figs 1 to 6). The ADP concentration in cultures of arginine-requiring organisms was directly proportional to the number of c.f.u. (ml culture)\(^{-1}\) (Figs 4 and 5).

At the mid-exponential growth phase, the mean ATP concentration ranged from 2.2 to 9.1 nmol ATP (mg dry weight)\(^{-1}\) in the four *Mycoplasma* spp., while in the two *Acholeplasma* spp. it was 8.4 and 15.6, (Table 1). At the same time, the mean EC\(_A\) of *A. laidlawii* was 0.90 ± 0.02 but that of other Mollicutes was significantly lower (\(P < 0.02\); mean EC\(_A\) 0.70 ± 0.06).

In order to determine whether lower values of ATP were caused by the turnover of ATP to ADP during the extraction of adenylates, samples of *M. gallisepticum* taken during the mid-exponential phase of growth were held at room temperature for 5 s, 1 min or 10 min before being transferred to Tris buffer at 95 °C. The respective ATP and ADP concentrations (nmol ml\(^{-1}\)) were 2.60 and 2.35 after 5 s, 2.80 and 2.50 after 1 min and 2.74 and 2.80 after 10 min. These values indicated that the low EC\(_A\) values reported above were unlikely to be due to rapid turnover of ATP to ADP during the extraction process.
Table 1. Growth rate and energy values of Mollicutes at mid-exponential growth phase

Except for E. coli, all the doubling time, ECₐ, and ATP values are computed from the statistically derived curves presented in Figs 1 to 6. All of the nucleotide values are considered to be cellular (see Discussion).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Doubling time (h)</th>
<th>ECₐ</th>
<th>Percentage ATP</th>
<th>Amount ATP [nmol (mg dry cell wt)-1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. laidlawii B-PG9</td>
<td>0.95</td>
<td>0.90</td>
<td>97</td>
<td>15.6 ± 0.28</td>
</tr>
<tr>
<td>A. morum S2</td>
<td>0.34</td>
<td>0.72</td>
<td>47</td>
<td>8.4 ± 0.84</td>
</tr>
<tr>
<td>M. gallisepticum S6</td>
<td>1.21</td>
<td>0.59</td>
<td>57</td>
<td>3.4 ± 0.72</td>
</tr>
<tr>
<td>M. fermentans PG18</td>
<td>0.63</td>
<td>0.74</td>
<td>54</td>
<td>9.1 ± 2.56</td>
</tr>
<tr>
<td>M. bovis PG45</td>
<td>1.86</td>
<td>0.76</td>
<td>63</td>
<td>6.9 ± 0.24</td>
</tr>
<tr>
<td>M. arginini G230</td>
<td>3.29</td>
<td>0.69</td>
<td>45</td>
<td>2.2 ± 0.17</td>
</tr>
<tr>
<td>E. coli ATCC 29522§</td>
<td>ND</td>
<td>0.89</td>
<td>92</td>
<td>9.5 ± 1.7</td>
</tr>
</tbody>
</table>

ND, Not determined.
* Calculated from c.f.u. determinations.
† Calculated as (nmol cellular ATP/nmol total adenylate nucleotide) × 100.
‡ Data in this column represent the amount of ATP related to the total dry cell weight (mass) from which it was extracted. The values are the means of data obtained from three to seven separate batches of cells ± s.d.
§ ATP was determined by the slower centrifugation-extraction-hexokinase method (Beaman & Pollack, 1981). All the values are the means of data from three separate batches of cells.

DISCUSSION

The utilization of glucose by M. arginini has not been previously reported. This organism has been thought to gain most if not all of its energy by metabolism of arginine through the arginine dihydrolase pathway (Barile et al., 1966). In this organism, Fenske & Kenny (1976) failed to detect, during early growth, the presence of arginine deiminase, the first enzyme in the pathway. Their work suggested that there was an alternative non-arginine-requiring pathway necessary for the production of energetically useful intermediates. That opinion is supported by our findings.

Since we did not separate cells from medium at the time of sampling as we did in an earlier study (Beaman & Pollack, 1981), we cannot report our data as that of cellular adenylate nucleotides; however, the increase in adenylate nucleotides is clearly the result of their net synthesis by the Mollicutes. We assume that the adenylate nucleotide data in Table 1 relate to cellular content, because in other experiments, at the mid-exponential growth phase of A. laidlawii B-PG9 and M. gallisepticum S6, cell-free culture filtrates contained less than 750 pmol of total adenylate nucleotide ml⁻¹ (J. D. Pollack, unpublished data). At the same period, the culture (medium plus cells) contained 10 to 13 nmol total adenylate nucleotide ml⁻¹ (Figs 1 and 3). Therefore, we assume that at the mid-exponential growth phase of A. laidlawii B-PG9 and M. gallisepticum S6, and with other Mollicutes in the same circumstances, at least 92 to 94% of the total culture adenylate nucleotide is cellular.

The use of the rapid extraction technique with A. laidlawii B-PG9 (Fig. 1) produced results that were essentially identical to those we have reported using the slower centrifugation-extraction-hexokinase technique (Beaman & Pollack, 1981). That is, for A. laidlawii B-PG9, the ECₐ remained high and relatively constant during the exponential growth phase and decreased during the stationary growth phase. However, as we found previously, the mean ECₐ values during the mid-exponential growth phase of M. gallisepticum and of other Mollicutes were significantly lower (about 20%; P < 0.02). These values are in marked contrast to those reported for Spiroplasm citri (Saglio et al., 1979) and a large number of bacteria (Karl, 1980), and to those we have detected in A. laidlawii B-PG9 by two procedures. This difference was also reflected during the same period by the mean percentage of ATP in the total adenylate nucleotide pool, which in A. laidlawii was 97%, and in E. coli 92%. In the remaining group of Mollicutes, the mean values were from 45 to 63%. Similarly, the mean mass of ATP (mg dry cell weight)⁻¹ was...
In other Mollicutes the mean values were lower ($P < 0.01$), ranging from 2.2 to 9.1. Apparently, apart from A. laidlawii B, the Mollicutes are in a state of relative energy deficit during their mid-exponential growth phase. We speculate further that this putative energy deficit of Mollicutes is related, with the probable exception of A. laidlawii B, to their generally vexatious association with eukaryotic cells and concomitant cytopathic effects. This hypothesis is supported by the work of Gabridge & Polisky (1977) with Mycoplasma pneumoniae. These workers found that tissue cultures infected with virulent M. pneumoniae had decreased ATP levels compared with tissue cultures infected with A. laidlawii or avirulent M. pneumoniae. In other studies, they were able to show that the addition of ATP or adenine decreased the pathogenic effects of M. pneumoniae in tissue culture (Gabridge & Stahl, 1978). Feldner et al. (1981) further demonstrated that attachment of M. gallisepticum to glass and to cells was dependent on ATP production. Our studies suggest that some Mollicutes, which like rickettsias and chlamydias are also deficient in cytochrome pigments, may to some degree be energy parasites in vivo. The lowered $E_C$ values in the four Mycoplasma species and A. morum possibly reflect some metabolic deficiency associated with their nucleic acid metabolism (Pollack & Hoffmann, 1982), or perhaps the relatively low concentrations of cellular ATP relate to insufficient substrate level phosphorylation linked to glycolysis (Pollack, 1981).

In preliminary studies, we found that $A. laidlawii$ B-PG9 has phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxytraphosphorylase activities, but that A. morum, M. gallisepticum, M. arginini, and M fermentans do not (K. D. Beaman & J. D. Pollack, unpublished results). This observation and the relatively higher $E_C$ and cellular ATP levels in $A. laidlawii$ B-PG9 reported in this work support our hypothesis that this organism is metabolically distinguishable from other Mollicutes.

REFERENCES


Pollack, J. D., Merola, A. J., Platz, J., & Booth,


