Differences in Accumulation of Radiolabeled Amino Acids and Polyamines by *Mycoplasma* and *Acholeplasma* Species

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Four species in the order *Mycoplasmatales*, *Mycoplasma capricolum*, *Mycoplasma hominis*, *Mycoplasma arginini*, and *Acholeplasma laidlawii*, were compared for their ability to accumulate radiolabeled amino acids and polyamines. The use of a novel high-molecular-weight (HMW) medium, from which molecules of less than 12,000 molecular weight had been removed by extensive dialysis, allowed us to discern significant differences among the species in their relative accumulations of [³H]methionine and [³H]leucine and of [³H]spermidine and [³H]putrescine. Accumulation of radiolabeled amino acids in control low-molecular-weight (LMW) medium was small (0.2 to 2% of the label), and the species did not differ in their proportional accumulations of methionine and leucine. Accumulation of methionine was significantly enhanced (5- to 12-fold) in all species in HMW medium. In contrast, leucine accumulation was enhanced sevenfold for *A. laidlawii* but only twofold for *M. hominis* and *M. capricolum* in HMW medium. The nonglycolytic species, *M. arginini*, accumulated radiolabeled putrescine and spermidine in both media, whereas the glycolytic species, *M. capricolum* and *A. laidlawii*, accumulated only radiolabeled spermidine. The ability to accumulate putrescine appeared to be a differential characteristic for nonfermentative, arginine-utilizing mycoplasmas. HMW medium was much more effective than LMW medium for use in radiolabeling *M. capricolum* proteins with [³⁵S]methionine.

As the smallest self-replicating procaryotes (300 to 500 nm), mycoplasmas are fastidious organisms that require complex cultivation media or host materials for growth and reproduction. Most mycoplasmas are cultured in an extract of yeast extract and exogenous sterols has become an important principle for supplies sterols, nucleic acids, amino acids, and peptides, as well as unspecified growth factors. The requirement for exogenous sterols has become an important principle for classification of mycoplasmas (16), while the ability of some species to accumulate and catabolize arginine via the arginine dihydrolase pathway has been used to separate mycoplasmas into glycolytic and arginine-utilizing species (2). In spite of the dependence of mycoplasmas upon external sources for virtually all of the amino acids (13, 15, 18, 25), little research has been done to investigate differences in amino acid accumulation and utilization among mycoplasmas, in part because of difficulties encountered in metabolically labeling these organisms with radioactive amino acids (21). Labeling difficulties have been ascribed to competition from free amino acids present in the cultivation media. Similarly, the procurement of polyamines (which are amino acid derivatives) by mycoplasmas has not been addressed. Although no evidence of polyamine synthesis from radiolabeled arginine, orthinine, or methionine was found in any of the mycoplasmas and acholeplasmas tested, all species had large intracellular pools of putrescine or spermidine or both (K. E. SJÖSTRÖM, Ph.D. dissertation, University of Washington, Seattle, 1990). The intracellular polyamines must therefore have been derived from the medium.

To increase the efficiency of radiolabeled amino acid accumulation, we employed a novel high-molecular-weight (HMW) medium from which free amino acids and other small molecules were removed by exhaustive dialysis. Using this HMW medium and a low-molecular-weight (LMW) medium as a control, we compared accumulation of the amino acids methionine and leucine and the polyamines spermidine and putrescine in *Acholeplasma laidlawii, Mycoplasma capricolum, Mycoplasma hominis,* and *Mycoplasma arginini*. Accumulation and incorporation were greatly enhanced in HMW medium.

**MATERIALS AND METHODS**

Preparation of media. The HMW medium fraction was prepared by dissolving 20 g of soy peptone powder and 10 g of yeast extract in 1 liter of H₂O and dialyzing for 72 h against six changes (total, 42 liters) of distilled H₂O. Standard cellulose dialysis tubing with a molecular weight cutoff of 12,000 to 14,000 (Spectrum Medical Industries, Los Angeles, Calif.) was used. The dialyzed solution was removed from the casing, prefiltered through a membrane (mean pore diameter, 800 nm) and filter sterilized by using a 220-nm-pore-size membrane (Millipore Corp., Bedford, Mass.). Filter sterilization was employed to avoid autoclaving and subsequent heat-generated hydrolysis of peptides. Whole horse serum (GIBCO Laboratories, Grand Island, N.Y.) was similarly dialyzed and sterilized. The LMW medium fraction was prepared by dissolving 20 g of soy peptone powder and 10 g of yeast extract (Difco Laboratories, Detroit, Mich.) in 200 ml of distilled H₂O and dialyzing the solution against 1 liter of distilled H₂O for 72 h at 4°C (14). Contents of the dialysis tubing were discarded, and the dialysate was sterilized by autoclaving. Each medium had a pH of approximately 7.1 after sterilization. LMW and HMW media were supplemented with 0.5% NaCl, 20 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer (pH 7.3), and 100 U of penicillin per ml. For most experiments, LMW medium contained 10% whole horse serum, and the HMW medium contained 10% dialyzed horse serum; for some experiments, gamma globulin-depleted horse serum (Alpha Gamma Labs, Sierra Madre, Calif.) was used instead. Glucose (2.5 mM) was added to HMW media only when specified. Complete media were tested for the

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presence of free amino acids and polyamines by two-dimensional thin-layer chromatography of dansylated derivatives on polyamide sheets, as previously described (5, 23). Briefly, 10 µl of each medium, containing approximately 50 µg of protein, was mixed with 40 µl of 1 M NaHCO₃ and 50 µl of dansyl chloride solution (5 mg/ml of acetone). After incubation at 37°C for 1 h followed by brief centrifugation, 5 µl of each supernatant was separated by thin-layer chromatography in two solvent systems. Location and concentration of amino acids and polyamines were determined by comparison with dansylated standards both visually and by measurements on a Shimadzu CS9000 scanning densitometer by using reflection mode at a wavelength of 340 nm.

**Culture of organisms.** Species used were *M. capricolum* kid (obtained from J. Tully [29]), *M. hominis* GX55-1 (23), *A. laidlawii* ATCC 14192 (10), and *M. arginini* G-230 (30) (obtained from M. Barile). Cultures (10 to 100 ml) were grown in LMW medium supplemented with 10% horse serum and washed two times at room temperature in HMW medium, and inoculated into LMW and HMW medium at a final concentration of approximately 10⁶ CFU/ml for at least four passages before use in these experiments. Organisms were harvested by centrifugation, washed two times at room temperature in HMW medium, and washed two times at room temperature in HMW medium, and inoculated into LMW and HMW medium at a final concentration of approximately 10⁶ CFU/ml for growing curves. For colony enumeration, organisms were inoculated onto H-agar plates.

**Radioactive labeling of organisms.** l-[methyl-³H]methionine (specific activity, 7.46 GBq/mmol), l-[4,5-³H(N)] leucine (185.0 GBq/mmol), l-[³⁵S]methionine (41.96 TBq/mmol), [terminal methylenes-³H(N)]permeidine trihydrochloride (161.7 TBq/mmol), and [2,3-³H(N)]putrescine dihydrochloride (111.0 TBq/mmol) were obtained from Dupont, NEN Research Products (Boston, Mass.) and used at an activity of 37 kBq/ml of medium (approximately 10⁴ dpm). Organisms (10 to 100 ml) were harvested in late log phase by centrifugation for 30 min at 10,000 x g. Intact harvested cells were washed twice in HMW medium for 10 min at room temperature to remove any adsorbed free amino acids. Cells were suspended in 10 ml of HMW medium to a concentration of approximately 10⁶ CFU/ml. In triplicate tubes, 0.1 ml of this suspension was added to 0.9 ml of HMW or LMW medium containing radiolabeled substrate and incubated at 37°C for 24 h. Hanks balanced salt solution (7) supplemented with 10% dialyzed horse serum was also used as a labeling medium for *M. capricolum*. Samples were pelleted for 5 min in an Eppendorf model 5414 microcentrifuge and washed twice in medium containing excess (50 mM) unlabeled substrate. The pellets were dissolved in 0.5 ml of Protosol (NEN) for 15 min at room temperature, suspended in 3 ml of scintillation fluid, and counted in a Beckman 3800 liquid scintillation counter. To determine incorporation into the acid-insoluble fraction, the pellets were precipitated with NEN Research Products (Boston, Mass.) and used at an amount by either *M. hominis* or *A. laidlawii* (Table 1).

**RESULTS**

**Comparison of HMW and LMW media.** When equivalent amounts (2.5 µg of protein) of the HMW and the LMW media were separated by thin-layer chromatography on polyamide sheets (Fig. 1), the fluorescence-labeled free amino acids in the HMW medium were at least 100 times less concentrated than those in the LMW medium, indicating that a significant proportion of these molecules had been removed by dialysis. Densitometry measurements of the chromatograms compared with known concentrations of dansylated substrates showed that methionine was present at a concentration of approximately 300 µM in HMW medium (Fig. 1A), but methionine was not detectable in HMW medium (concentration >1 µM) (Fig. 1B). Less than 30 µM leucine remained in the HMW medium after dialysis, compared with more than 3 mM leucine in the LMW medium. Putrescine and spermidine were not detectable in either medium. The dansyl hydroxide spot was equally fluorescent in both chromatograms, showing that the same sample load was applied to each sheet.

**Accumulation of amino acids.** When mycoplasmas were incubated in LMW medium supplemented with radiolabeled substrate, methionine and leucine were accumulated equally well by either *M. hominis* or *A. laidlawii* (Table 1). *M. capricolum* showed a slightly greater accumulation of radiolabeled methionine (1.3 times more methionine than leucine). In HMW labeling medium, however, both *M. capricolum* and *M. hominis* accumulated a greater proportion of labeled methionine than of leucine (nine times more for *M. capricolum* and six times more for *M. hominis*), whereas *A. laidlawii* accumulated twice the proportion of labeled leu-
cine. Nearly 24% of the available radiolabeled methionine was found in the M. capricolum pellet after incubation in HMW labeling medium, compared with only 2% in the LMW medium, a 12-fold increase in accumulation. When the accumulation of tritiated amino acids was calculated on a cellular basis, a 300-fold increase in radiolabeled methionine accumulation was observed with 3.2 pM unlabeled methionine, and a 90% reduction in LMW medium by a factor of 1.5 but had no effect in LMW medium. Passage of M. capricolum in HMW medium prior to labeling experiments had no effect on the effect in LMW medium. Passage of M. capricolum of an identical gel showed much more intense labeling of proteins from M. hominis. Autoradiography of an identical gel showed much more intense labeling of proteins from M. capricolum grown in HMW medium (Fig. 2). Equal volumes of radiolabeled cell suspensions from LMW and HMW media (containing approximately 70 and 30 μg of protein, respectively), after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining by Coomassie blue, showed identical polypeptide patterns. Autoradiography of an identical gel showed much more intense labeling of proteins from M. capricolum grown in HMW medium (Fig. 2, lane D) even though the total protein load was lower in the HMW-medium lane. Similar results were obtained with M. hominis (data not shown).

**Growth of mycoplasmas in HMW medium.** In HMW me-

### TABLE 1. Accumulation of radiolabeled methionine and leucine by mycoplasmas in LMW and HMW media

<table>
<thead>
<tr>
<th>Species</th>
<th>Substrate</th>
<th>% Accumulation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio H/L&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Cellular accumulation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ratio H/L&lt;sup&gt;c&lt;/sup&gt;</th>
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<td></td>
<td>LMW</td>
<td>HMW</td>
<td></td>
<td>LMW</td>
</tr>
<tr>
<td>M. hominis</td>
<td>[3H]methionine</td>
<td>0.15</td>
<td>1.56</td>
<td>10</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>[3H]leucine</td>
<td>0.14</td>
<td>0.25</td>
<td></td>
<td>122</td>
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<tr>
<td>M. capricolum</td>
<td>[3H]methionine</td>
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<td>23.75</td>
<td>12</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>[3H]leucine</td>
<td>1.48</td>
<td>2.72</td>
<td>2</td>
<td>144</td>
</tr>
<tr>
<td>A. laidlawii</td>
<td>[3H]methionine</td>
<td>0.30</td>
<td>1.54</td>
<td>5</td>
<td>299</td>
</tr>
<tr>
<td></td>
<td>[3H]leucine</td>
<td>0.37</td>
<td>2.90</td>
<td>7</td>
<td>425</td>
</tr>
</tbody>
</table>

<sup>a</sup> Dpm in a washed pellet from 1 ml of culture, expressed as the percent dpm in 1 ml of uninoculated culture medium.

<sup>b</sup> Cellular accumulation was defined as dpm/10<sup>6</sup> CFU.

<sup>c</sup> Ratio of label accumulation in HMW medium to label accumulation in LMW medium. All ratios were significantly different (P < 0.01).

### TABLE 2. Accumulation of amino acids on a cellular basis

<table>
<thead>
<tr>
<th>Species</th>
<th>Substrate</th>
<th>% Accumulation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio H/L&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Cellular accumulation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ratio H/L&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>LMW</td>
<td>HMW</td>
<td></td>
<td>LMW</td>
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<td>[3H]methionine</td>
<td>0.15</td>
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<sup>a</sup> Dpm in a washed pellet from 1 ml of culture, expressed as the percent dpm in 1 ml of uninoculated culture medium. All values are averages of triplicate samples from two experiments.

<sup>b</sup> Cellular accumulation was defined as dpm/10<sup>6</sup> CFU.

<sup>c</sup> Ratio of label accumulation in HMW medium to label accumulation in LMW medium. All ratios were significantly different (P < 0.01).
dium, M. capricolum and A. laidlawii showed a longer lag phase than that found in LMW medium (Fig. 3A), and the resulting CFU per milliliter were approximately 10-fold lower in HMW medium for both species than in the LMW medium. Neither M. hominis nor M. arginini showed an increase in CFU per milliliter when incubated in HMW medium over a 4-day period, but they remained viable at the same population level for at least 72 h, as shown by colony counts (Fig. 3B). Inclusion of 2.5 mM glucose in HMW medium did not affect rates of growth or final CFU per milliliter for M. capricolum or A. laidlawii, although cultures became more acidic than in medium without glucose.

Controls. When washed pellets were precipitated with trichloroacetic acid, 60 to 70% of the amount of radiolabel found with Protosol extraction was recovered. Samples incubated at 4°C for 24 h showed no uptake of label into the pellet. Low residual radioactivity (50 to 100 cpm/ml) was found on washed plastic tubes which had been incubated at 37°C with labeling media but no organisms.

**DISCUSSION**

The accumulation of radiolabeled amino acids by mycoplasmas was considerably facilitated by the use of a HMW medium fraction from which small molecules, oligopeptides, and free amino acids had been removed by extensive dialysis. Accumulation of radiolabel was significantly better in this medium than in an LMW medium; these results are similar to those found for trichomonads, which are cultured in a medium quite similar to that used for mycoplasmas (28). Incubation in LMW medium produced little detectable difference in the proportional accumulation of radiolabeled methionine and leucine by M. capricolum, M. hominis, or A. laidlawii. In HMW medium, however, M. capricolum and M. hominis accumulated higher levels of radiolabeled methionine than radiolabeled leucine, while A. laidlawii accumulated a higher proportion of labeled leucine.

Accumulation of tritiated methionine by M. capricolum in HMW medium was reduced 90% by adding a low concentration (32 μM) of unlabeled methionine to the medium. A plateau level of accumulation (2%) was reached at 100 μM methionine, well below the concentration of methionine found in LMW medium (300 μM). This evidence suggests that methionine was taken up as a free amino acid by M. capricolum. Similarly, Razin et al. found that the uptake of radiolabeled methionine by M. hominis was strongly inhibited by unlabeled free methionine (17). By comparison, 3.2 mM unlabeled leucine was required to produce a similar reduction in tritiated leucine accumulation. The concentration of residual leucine in the HMW medium (30 μM) was much less than the concentration of unlabeled substrate required for the 90% reduction of label accumulation, suggesting that the relatively small uptake of radiolabeled leu- cine in both media was not simply a result of competition from unlabeled free leucine. It is possible that leucine is accumulated preferentially as part of a larger molecule by M. capricolum.

The ability of polypeptides to stimulate growth in mycoplasmas has been documented (18, 26). Our strain of M. capricolum has an intracellular protease capable of cleaving leucine residues (Sjöström, Ph.D. dissertation), so the prime

### Table 3. Accumulation* of radiolabeled spermidine and putrescine by mycoplasmas in LMWb and HMWc media

<table>
<thead>
<tr>
<th>Species</th>
<th>Substrate</th>
<th>% Accumulationa</th>
<th>Ratio of spd/putd</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. hominis</td>
<td>[3H]spermidine</td>
<td>4.85</td>
<td>1 (NS)</td>
</tr>
<tr>
<td></td>
<td>[3H]putrescine</td>
<td>4.26</td>
<td>1 (NS)</td>
</tr>
<tr>
<td>M. arginini</td>
<td>[3H]spermidine</td>
<td>5.20</td>
<td>1 (NS)</td>
</tr>
<tr>
<td></td>
<td>[3H]putrescine</td>
<td>5.05</td>
<td>1 (NS)</td>
</tr>
<tr>
<td>M. capricolum</td>
<td>[3H]spermidine</td>
<td>9.80</td>
<td>39 (P &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>[3H]putrescine</td>
<td>0.20</td>
<td>40 (P &lt; 0.001)</td>
</tr>
<tr>
<td>A. laidlawii</td>
<td>[3H]spermidine</td>
<td>3.52</td>
<td>12 (P &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>[3H]putrescine</td>
<td>0.30</td>
<td>14 (P &lt; 0.001)</td>
</tr>
</tbody>
</table>

* Dpm in a washed pellet from 1 ml of culture, expressed as the percent dpm in 1 ml of uninoculated culture medium.

b <14,000.

c >14,000.

d Ratio of spd/put was defined as accumulation of [3H]spermidine/accumulation of [3H]putrescine. Not significant (P > 0.05).

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FIG. 2. Radiolabeling of mycoplasmal proteins. M. capricolum was grown in LMW medium (lanes B and D) or HMW medium (lanes C and E) containing 37 KBq/ml l-[35S]methionine. Concentrated organisms (75 μl) were added to each lane of a sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, one-half of the gel was stained for protein, while the other half was exposed to X-Omat film (Kodak) for 48 h at −70°C. Lanes: A, prestained molecular weight markers (Sigma Chemical Co., St. Louis, Mo.), 38 μg of total protein; B, Coomassie blue-stained protein bands from LMW medium, 70 μg of protein; C, Coomassie blue-stained protein bands from HMW medium, 30 μg of protein; D, autoradiogram of protein bands from LMW medium, 70 μg of protein; E, autoradiogram of protein bands from HMW medium, 30 μg of protein. kDa, Kilodaltons.
source of leucine might be a peptide which is more readily transported than free leucine. The amino acid nutrition of mycoplasmas is complex, since antagonisms are observed between amino acids and since certain small peptides appear to have an important function in the supply in amino acids (26). The ability of peptides to enhance growth, even in the presence of an entire complement of free amino acids, led Smith (26) to postulate a nutritional need in mycoplasmas for small peptides, possibly as an assimilable source of amino acids and for neutralization of fatty acid toxicity. Some gram-positive bacteria also utilize peptides: peptides were taken up in preference to free amino acids by Lactobacillus casei (12). The presence of peptides enhanced uptake of radiolabeled amino acids in streptococci (4) by a suggested mechanism in which peptides were taken up by a transport system distinct from that for amino acids, the peptides hydrolyzed intracellularly, and the surplus amino acids then exchanged for essential amino acids by a porter-transporter system.

M. capricolum and A. laidlawii grew well in HMW medium, indicating that these two species have the proteolytic enzymes necessary to obtain the amino acids required for protein synthesis from the large proteins and polypeptides in the HMW medium. This result is not surprising, since Rodwell (19) has indicated that Mycoplasma mycoides subsp. mycoides Y (a species closely related to M. capricolum and with similar nutritional requirements) can obtain most of its amino acid needs from bovine serum albumin in a partially defined medium and A. laidlawii is known to possess peptidase activity (6). On the other hand, the two nonfermentative, arginine-utilizing organisms, M. hominis and M. arginini, survived for over 3 days in the HMW medium but did not grow. As arginine-utilizing species also have proteolytic enzymes (1, 22), their inability to proliferate may indicate a need for critical small molecules not present in the HMW medium. M. hominis and M. arginini were able to accumulate radiolabeled amino acids and polyamines during incubation in HMW medium, suggesting that some metabolic functions were supported in this medium. M. capricolum rapidly died in balanced salt solution with serum and showed no radiolabel incorporation, indicating that this was not a satisfactory labeling medium. Overall, even though the total yield of culture was less in HMW medium, improved labeling was observed even for those species which did not replicate in this medium. Results of trichloroacetic acid extraction, as well as autoradiograms, showed incorporation of $L^{35}$S methionine into specific mycoplasmal proteins.

Another significant finding was the ability of nonglycolytic arginine-utilizing species such as M. hominis and M. arginini to accumulate putrescine and spermidine under conditions in which glycolytic species accumulated only spermidine. Competition experiments demonstrated that spermidine and putrescine were accumulated by two distinct systems in M. hominis, since 100 times as much of the heterologous polyamine was required for equivalent reduction in uptake. The system for spermidine accumulation in M. capricolum was highly specific for spermidine: unlabeled spermidine, at the 0.032 mM level, reduced radiolabeled spermidine accumulation by 90%, whereas 300-fold more putrescine (which is accumulated only negligibly by M. capricolum) reduced spermidine uptake by only 40%. In Escherichia coli, spermidine and putrescine are also accumulated by separate transport systems (24, 27). Herbst et al. (8) found that gram-positive bacteria generally had spermidine as the major polyamine component, with little or no detectable putrescine, whereas gram-negative species tended to have high levels of putrescine but no spermidine. E. coli is the exception which contains both spermidine and putrescine. Mycoplasmas have been shown to be related to the Bacillus-Lactobacillus-Streptococcus subgroup of gram-positive bacteria through analysis of 16S rRNA oligonucleotides (31). The ability demonstrated by all mycoplasmas tested to take up spermidine is consistent with their gram-positive heritage. The uptake of putrescine by nonfermentative arginine-utilizing species may represent a unique nutritional requirement of these species which may be taxonomically useful.

ACKNOWLEDGMENTS

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LITERATURE CITED


