DEFINED MEDIUM FOR MYCOPLASMA LAIDLAWII

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ABSTRACT

TOURTELLOTTE, MARK E. (University of Connecticut, Storrs), HAROLD J. MOROWITZ, AND PHIL KASIMER. Defined medium for Mycoplasma laidlawii. J. Bacteriol. 88:11-15. 1964.—A defined medium for the pleuropneumonia-like organism Mycoplasma laidlawii B is described in which absolute requirements for growth in the basal medium were obtained in the presence of two purified peptides from crystalline ribonuclease, one of which has the amino acid sequence threonine-threonine-glutamine-alanine-asparagine-lysine, and the other lysine-glutamic acid-threonine-alanine-alanine-lysine-lysine. Continuous, but suboptimal, growth was obtained with the single ribonuclease peptide: lysine-glutamic acid-threonine-alanine-alanine-lysine.

The organisms of the pleuropneumonia group (genus Mycoplasma) appear to be exacting in their nutritional requirements (Razin, 1962). Most strains require complex media consisting of meat infusions and sera. Attempts to grow pleuropneumonia-like organisms (PPLO) in a completely defined medium have so far been unsuccessful. Smith (1955), Rodwell (1960), Razin and Knight (1960), and Razin and Cohen (1963) obtained growth in otherwise defined medium only upon the addition of components such as lipoprotein, heat-stable protein, or bovine serum albumin.

This paper reports on the development of a defined medium for the saprophytic PPLO M. laidlawii B, in which such ill-defined substances as serum and enzymatic digests of meat were replaced by amino acids, nucleosides, vitamins, and purified peptides of known amino acid sequence.

MATERIALS AND METHODS

Strain. M. laidlawii strain B was maintained in Tryptose Broth (Difco) supplemented with 1% PPLO Serum Fraction (Difco) and 1% glucose (Morowitz et al., 1962).

Reagents. The reagents used in the defined medium were of highest purity commercially available. Crystalline trypsin (2X), crystalline ribonuclease (5X), and the oxidized ribonuclease were obtained from Mann Research Laboratory, New York, N.Y. Oleic acid-1-C14 and linoleic acid-1-C14 were obtained from Calbiochem. Stock solutions of chemicals were prepared in distilled water and sterilized by filtration through sintered glass or Millipore filters (0.45 μm).

Glassware. Glassware was either placed in a solution of potassium dichromate-sulfuric acid for 24 hr and thoroughly rinsed with distilled water, or washed in “7X” (Linbro Chemical Corp., New Haven, Conn.) and rinsed.

Peptides. A lyophilized preparation of performic acid oxidized ribonuclease was dissolved in distilled water to make a 1% solution. The solution was adjusted to pH 8.0 with sodium hydroxide. Trypsin was added to a final concentration of 0.01%. Digestion then took place at room temperature, with periodic additions of NaOH to keep the solution at pH 8.0. When the reaction was complete, as judged by the cessation of acid production, the solution was boiled for 20 min to destroy proteolytic activity. The precipitated material was removed by filtration, and the filtrate was lyophilized and redissolved in pyridine-glacial acetic acid-water (50:2:950) at pH 6.5. This digest was separated into three fractions (positive, negative, and neutral) by hanging-curtain electrophoresis in a Beckman-Spinco model CP continuous-flow paper electrophoresis cell. The positive fraction was further separated...
into peptides by hanging-strip electrophoresis (pH 6.5) in a Beckman-Spinco model R paper electrophoresis cell and chromatography with n-butanol-acetic acid-water-pyridine (30:6:24:20). Tryptic peptides of trypsin were prepared in a similar manner with autoclaved trypsin as starting material.

_Uptake of C₁₄-labeled fatty acids._ Oleic acid-1-C¹⁴ and linoleic acid-1-C¹⁴ (5 μc of each) were added separately to cell suspensions in Tryptose Broth and incubated for 1 hr at 37 C. Cells were centrifuged from the medium at 4 C and immediately extracted with chloroform-methanol (2:1). The lipid extract was evaporated to dryness under a stream of N₂ at 37 C. Lipids were fractionated and identified, and radioactivity was determined, by methods previously described (Tourtelotte et al., 1963).

_Growth conditions._ Cultures were incubated in tubes at 37 C without agitation. Assessment of growth was by turbidity (optical density at 420 μm) and by viable counts (Cleverdon and Morowitz, 1960).

**Results**

In attempts to determine the nutritional requirements for _M. laidlawii_ B, the tissue culture media TC199, TC858, and TC Medium NCTC 107 (Difco) were initially tried but did not support growth. Good growth of this strain was obtained with a medium similar to that described by Razin and Knight (1960), in which deoxyribonucleosides and ribonucleosides were substituted for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), and 1% PPLO Serum Fraction for horse serum.

By various modifications of this medium, a medium which gave growth equivalent to that obtained in Tryptose Broth containing 1% PPLO Serum Fraction was obtained (Table 1).

_Inorganic salts._ Trace minerals and ash from peptone (Difco) were added, but were subsequently found to be nonessential for growth. Because this organism produces acid from glucose which rapidly kills the organisms, and because phosphate at high concentrations was found to be toxic, tris [2-amino-2-(hydroxymethyl)-1,3-propanediol] was added as buffer.

_Vitamins._ Choline-HCl, folic acid, inositol, and calcium pantothenate were not required for growth. Omission of coenzyme A (CoA) resulted in no growth, and could not be replaced by calcium pantothenate and adenosine mono-, di-, or triphosphate. Pantotheine was not tested. Addition of acetate in the absence of CoA did not result in increased growth.

_Lipids._ When PPLO Serum Fraction was added to the basal medium, fatty acids were not required, the acids being supplied in this case by the lipids present in the serum lipoprotein.

Although five fatty acids, oleic, palmitic, myristic, linoleic, and linolenic, were required for optimal growth, oleic acid alone gave approximately half optimal growth. The concentration of fatty acids was important; if the concentration was reduced to 4 μg per liter or increased above 2 mg per liter, little or no growth occurred. Incubation of cells in the presence of oleic acid-1-C¹⁴ and linoleic-1-C¹⁴ resulted in a rapid uptake of these fatty acids. Separation of cellular lipids into neutral and phospholipids on silicic acid columns and fractionation of lipids showed most of the radioactive fatty acids to be esterified as di-glyceride or phospholipid, with very little existing as the free acids. Over 90% of the total radioactivity of the cells was in phospholipid.

Cholesterol and other lipids were not required for growth. Glycerol phosphate for phospholipid synthesis is presumably supplied by glucose, which was also found to be essential for good growth. As shown previously by Tourtelotte and Jacobs (1960), glucose is also essential as an energy source.

_Nucleic acids._ The nucleosides, thymidine, adenosine, cytidine, and guanosine, at a concentration of 5 mg per liter, support excellent growth. The riboside uridine and the deoxyribosides, deoxyguanosine, deoxyctydine, and deoxyadenosine, were not essential. Substitution of deoxyribosides for ribosides also resulted in optimal growth. Substitution of free bases for the deoxyribosides and ribosides, and addition of ribose and deoxyribose, supported growth which was delayed and never reached the optimal level. It is important to note, however, that continuous growth in medium containing free bases and pentose sugars did occur.

_Amino acids._ Because it has been difficult to obtain large quantities of purified ribonuclease peptide free from other peptides containing all the amino acids, the amino acid requirements have not yet been elucidated. However, there is an absolute requirement for cysteine and, although the results were somewhat variable, probably...
tryptophan. High concentrations (500 mg per liter) of histidine and aspartic acid were toxic.

The amino acids at the concentrations listed in Table 1 supported optimal growth, as compared with growth in Tryptose plus PPLO Serum Fraction (Difco). Casamino Acids (Difco, vitamin-free, acid-hydrolyzed casein) at a 1% concentration, plus cystine and tryptophan (10 mg per liter), effectively replaced the amino acids.  

**Table 1. Defined medium for Mycoplasma laidlawii B**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amt*</th>
<th>Component</th>
<th>Amt*</th>
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</thead>
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<tr>
<td>Arginine</td>
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<td>NaCl</td>
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</tr>
<tr>
<td>Phenylalanine</td>
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<td>KCl</td>
<td>400</td>
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<tr>
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<td>MgSO₄</td>
<td>200</td>
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<td>Na₂HPO₄</td>
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<tr>
<td>Alanine</td>
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<td>Thiamine</td>
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<td>Cystine</td>
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<td>Pyridoxine</td>
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</tr>
<tr>
<td>Glutamic acid</td>
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</tr>
<tr>
<td>Aspartic acid</td>
<td>4</td>
<td>Nicotinic acid</td>
<td>0.025</td>
</tr>
<tr>
<td>Glutamine</td>
<td>3</td>
<td>Biotin</td>
<td>0.025</td>
</tr>
<tr>
<td>Asparagine</td>
<td>3</td>
<td>Nicotinic acid amide</td>
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<td>Histidine</td>
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<td>Palmitic acid</td>
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</tr>
<tr>
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<td>Linoleic acid</td>
<td>0.4</td>
</tr>
<tr>
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<td>Linolenic acid</td>
<td>0.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>50</td>
<td>Myristic acid</td>
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<td>Proline</td>
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<td>Lysine-glutamic acid-threonine-alanine-alanine-lysine</td>
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<td>Adenosine</td>
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</tr>
<tr>
<td>Guanosine</td>
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<td></td>
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* Expressed as milligrams per liter of distilled water. Final pH was 8.0.  

Because of the limited yields of positive peptides, it has been difficult to carry out extensive chemical analyses. However, we obtained one fraction which contains two peptides, one of which was identified as Hirs "O-tryp-5" peptide, with the following amino acid sequence: threonine-threonine-glutamine-alanine-asparagine-lysine; the other was "O-tryp-10" (Hirs, 1960). This peptide mixture supported excellent growth of *M. laidlawii* B (Fig. 1). The amino acid com-
position of this mixture was determined on one sample. From the relative amounts of the different amino acids, the mixture appears to be 64% "O-tryp-8" and 36% "O-tryp-10."

A purified sample of "O-tryp-10" peptide which has the sequence lysine-glutamic acid-threonine-alanine-alanine-alanine-lysine, supplied by F. Richards (Yale University, New Haven, Conn.), supported growth through several continuous passages; however, growth was suboptimal and inferior to growth obtained with the aforementioned peptides.

Little or no growth was obtained with either raw or heat-denatured ribonuclease in concentrations ranging from 50 to 500 μg/ml. These findings indicate that the factor is a peptide and not traces of contaminating material in the ribonuclease.

In addition to peptides from ribonuclease, trypsic digests of heat-denatured trypsin and algal protein supported good growth. Attempts to replace this growth factor with protamine, glutathione, and numerous commercially available dipeptides were not successful. A synthetic peptide, seryl-histidyl-leucyl-valyl-glutamic acid, with high strepogenin activity, also failed to support growth of M. laidlawii.

**Discussion**

The development of a defined medium for the growth of one of the *Mycoplasma*, which contains no complex lipids or macromolecules such as nucleic acid, protein, or polysaccharide, provides a more rational basis for metabolic studies of *Mycoplasma* and answers several questions as to their synthetic ability. Thus, it is apparent that *M. laidlawii* does not require phospholipids, oligonucleotides, or high molecular weight protein, but possesses the enzymes capable of synthesizing these cellular components. The medium described here is not a minimal defined medium, and experiments are in progress to remove non-essential components.

Growth in this medium, which contains no lipid other than fatty acids, demonstrated that *M. laidlawii* does not require cholesterol for growth. This confirms reports by Smith (1960) that this organism will replicate in complex medium previously extracted with ether, and by Razin and Knight (1960) who obtained growth in a cholesterol-free partially defined medium.

A nutritional requirement for fatty acids, and the appearance of C18 oleic and linoleic acids in neutral lipids and phospholipids, clearly demonstrates that fatty acids are incorporated into cellular lipids. The finding that acetate does not replace fatty acids suggests that the organism is incapable of synthesizing long-chain fatty acid from acetate. Long-chain fatty acids appear to be true nutritional requirements, and do not function solely as surface tension depressants, as suggested by Rodwell (1960).

In light of the high lipid content of PPLO (Tourtellotte et al., 1963), the need for CoA for synthesis of these lipids is not unexpected. It is somewhat surprising, however, that CoA is not synthesized by this organism, because most heterotrophic bacteria are capable of synthesizing it from pantothenic acid and other precursors.

The minimal nucleic acid requirement for optimal growth of *M. laidlawii* B, namely, thymidine, adenosine, cytidine, and guanosine, agrees closely with the minimal requirements for *M. laidlawii* A reported recently by Razin and Cohen (1963).

Growth, though suboptimal, with the purines and pyrimidines, thymine, uracil, cytosine,

![Graph](http://jb.asm.org/Downloaded from http://jb.asm.org/)
adenine, and guanine, in the presence of ribose and deoxyribose, suggests that the organism has considerable synthetic ability in making nucleic acids.

The requirement of peptide for the growth of Mycoplasma immediately raises the questions as to its function. Although no extensive studies have as yet been made, numerous tryptic peptides will supply the factor; therefore, it would seem that \textit{M. laidlawii} does not require a peptide with a specific amino acid sequence. This is reminiscent of the strepogenin requirement for \textit{Lactobacillus casei} and the peptide requirement for \textit{L. bulgaricus}, in which numerous peptides with no common amino acid sequence stimulated growth (Jones and Woolley, 1962). Jones and Woolley (1962) showed that growth of \textit{L. bulgaricus} was stimulated by numerous peptides, the only amino acid in common being valine. However, other valine-containing peptides were inactive, and some peptides which supported growth contained no detectable valine.

Kihara and Snell (1960), working with a strain of \textit{L. casei} in which growth was stimulated by "streptogenin," were successful in replacing these peptides with a synthetic mixture of cysteine, glutamine, serine, aspartic acid, guanlyc acid, uracil, spermine, oleic acid, and Tween 40 in appropriate concentrations. From this work, they concluded that the peptide supplies several limiting amino acids in a form that can be absorbed and utilized by the cell more rapidly than the free amino acids in the medium. Whether the peptides required by \textit{M. laidlawii} serve a similar function awaits further research.

**Acknowledgments**

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**Literature Cited**


