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A strain of *Melissococcus pluton* cultivable on chemically defined media

(*Melissococcus pluton*; chemically defined media; honey bee)

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1. SUMMARY

A Brazilian strain of *Melissococcus pluton* multiplied well under anaerobic conditions on chemically defined media buffered with potassium phosphate. Thymine, xanthine, pyridoxal and one or more other vitamins, CO_2, glucose, methionine and possibly other amino acids, were essential. Other strains multiplied only slightly and could not be subcultivated on the media. The Brazil strain is the least related serologically to all known strains of *M. pluton* and is the least fastiduous when cultivated on other media. It has a DNA base composition of 31.4 G + C mol%, whereas others tested have been 29.0 and 30.2 G + C mol%.

2. INTRODUCTION

M. pluton [1,2], the cause of European foulbrood of bees, is a fastidious organism and has been cultivated only on media containing some chemically undefined nutrients. Recently, I have cultivated a strain of *M. pluton* on a chemically defined medium.

3. MATERIALS AND METHODS

3.1. Strains of M. pluton

Some of the 18 strains of *M. pluton*, isolated from honey bee larvae with European foulbrood from many parts of the world were used, and were maintained on the maintenance medium described below or in lyophilized form, using methods described by Bailey and Collins [1].

3.2. Media

The maintenance medium comprised (g/l): peptone (Oxoid L37) 10, cysteine 1, glucose 10, soluble starch 10, KH_2PO_4 13.7, agar 20, pH 6.6; autoclaved in sealed screw-capped bottles at 116 °C for 20 min and sloped for immediate use.

Two chemically defined media were devised, one being a simplified version of the other (Table 1). Their carbohydrates and salts were sterilized, at twice the given concentrations, together with agar (40 g/l), at 116 °C for 20 min. The rest of the constituents were sterilized, also at twice the given concentrations, by filtration, then mixed with equal volumes of the sterilized carbohydrates, salts and agar, and poured into petri dishes for immediate use.

Unless stated otherwise, cultures were incubated at 35 °C anaerobically in McIntosh and

Table 1

Chemically defined media

Amino acids	*g/l*	*Carbohydrates*	*g/l*
L-Alanine	0.2	Glucose	10
L-Arginine HCl	0.2	Soluble starch [a]	10
L-Asparagine	0.4	*Vitamins*	*μg/l*
L-Aspartic acid	0.1	*d*-Biotin [a]	2.0
L-Cysteine HCl [a]	0.5	Folic acid [a]	2.0
L-Cystine [a]	0.1	DL6,8-Thioctic acid [a]	200
L-Glutamic acid	0.4	Pyridoxal HCl	200
L-Glutamine	0.02	Pyridoxamine diHCl [a]	200
Glycine	0.1	*P*-aminobenzoic acid	200
L-Histidine HCl	0.05	*d*-Pantothenic acid, Ca salt	400
4 Hydroxy-L-proline	0.05	Niacinamide	400
L-Isoleucine	0.25	Riboflavin	200
L-Leucine	0.05	Thiamine HCl	400
L-Lysine HCl	0.1	Pyridoxine HCl [a]	200
L-Methionine	0.1	B_{12} [a]	2.5
L-Phenylalanine	0.1	*Bases*	*mg/l*
L-Proline	0.1	Uracil [a]	10
L-Serine	0.05	Thymine	10
L-Threonine	0.05	Guanine [a]	10
L-Tryptophan	0.05	Adenine [a]	10
L-Tyrosine	0.05	Xanthine	10
L-Valine	0.1	Cytosine [a]	10
L-Citrulline	0.2	*Organic acids* [a]	*mg/l*
DHP-alanine [a]	0.2	Malic	100
Salts	*g/l*	Succinic	100
$MgSO_4, 7H_2O$ [a]	0.2	Pyruvic, Na salt	100
$FeSO_4, 7H_2O$ [a]	0.01	Oleic, Na salt	200
$MnSO_4, 4H_2O$ [a]	0.01	*Others* [a]	*mg/l*
$CaCl_2, 6H_2O$ [a]	0.03	Hemin chloride	10
NaCl	0.6	Menadione	10
KH_2PO_4	13.7	Acetylcholine chloride	100
KOH	4.0	Choline chloride	100
		Myoinositol	10

[a] Not in the simplified medium.

Fildes jars containing hydrogen plus 5% (v/v) CO_2.

3.3. Serology and DNA base determinations

Antisera against the New York strain and the India F strains were prepared and assays made with them, as described by Bailey and Collins [1]. The New York antiserum 1 (Table 2) was probably the least specific because it was prepared by four intravenous injections whereas the others were each prepared by a single intramuscular injection.

DNA base determinations were done as described by Bailey and Collins [1].

4. RESULTS AND DISCUSSION

Strains UK, Tanzania and Brazil, which had been maintained in vitro for many years, were used for tests with chemically defined media. In many tests with the complete medium, the UK strain multiplied very slightly once and the Tanzania strain twice, but neither strain could be subcultivated on the medium. However, the Brazil strain multiplied reliably and well, and the simplified medium was devised by the use of this strain.

Methionine, glucose, thymine, xanthine, pyridoxal, one or more of the other vitamins of the simplified medium and CO_2 were essential. Cysteine or cystine would not replace methionine, but were beneficial in the presence of methionine. The effects of removing other amino acids were not tested.

Replacing the starch, salts and vitamin B_{12}, and adding taurine plus homocysteine (1 g/l of each) plus cystathionine (200 mg/l) did not make the simplified medium suitable for any strain other than Brazil.

The complete medium was made suitable for any strain by the addition of 1 to 3 g/l Yeast Extract (Difco), or 10 g/l peptone (Oxoid L37).

The Brazil strain was the least fastidious of all the known strains of *M. pluton*. Tests with 12 strains of *M. pluton* on plates of the maintenance medium ± soluble starch, scoring the amount of growth on a scale of 1 to 4, gave means of 3.2 ± 0.32 with starch and of 1.25 ± 0.39 without starch. The difference is statistically significant ($P < 0.02$). However, the Brazil strain did not require starch, and multiplication of the Tanzania strain was only slightly improved by it. The Brazil strain also would multiply, although weakly and only after a delay of several days, on the maintenance medium with sodium phosphate instead of potassium phosphate; and it multiplied slowly in litmus milk (incubated aerobically) causing coagulation, slight acidification and slight reduction after several days. The strain would also multiply on the maintenance medium with sucrose or melezitose instead of glucose, but no tests were done with these sugars on the other strains except UK, which failed to multiply.

Table 2

Agglutination tests of isolates of *Melissococcus pluton*

Isolate	Antiserum titre			
	New York strain antisera			Indian strain F antiserum
	1	2	3	
India F	128	–	–	128
New York	2048	512	128	–
UK	1024	128	128	256
Brazil	512	<1	16	8

The UK, Tanzania and Brazil strains multiplied equally, but only slightly, on an agar of 10 g peptone (Oxoid L37) + 10 g glucose/l under anaerobic conditions plus CO_2. However, when the peptone was replaced with Oxoid Nutrient Broth (No. 2), the Brazil strain multiplied fairly well, giving a final pH of 4.5, whereas the other strains failed to multiply. The Brazil strain also multiplied feebly and slowly on the maintenance medium in fully aerobic conditions whereas the UK and Tanzania strains multiplied similarly only in air plus 5 to 20% CO_2. No strain multiplied in the absence of CO_2 i.e. when enclosed in any atmosphere over dilute KOH.

The results of the serological assays are given in Table 2. This and the results of Bailey and Collins [1] show that the Brazil strain is least related to the rest.

The DNA base composition of the Brazil strain was 31.4 G + C mol%. Those of the four other strains tested were between 29.0 and 30.2 G + C mol% [1].

To sum up, the Brazil strain differs from the other known strains of *M. pluton,* but the differences are of degree rather than kind. Serology and G + C content separate it from the other strains tested, but it remains well differentiated from the genus *Streptococcus.* It may have little or only local significance in nature, but its behaviour in chemically defined media provides a guide for studies of the nutritional requirements of the genus.

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