Modification of the Membrane Composition of *Mycoplasma mycoides* subsp. *capri* by the Growth Medium

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(Received 20 November 1974; revised 22 January 1975)

SUMMARY

Mycoplasma mycoides subsp. *capri* was grown in different media. The amounts of the lipids in these media were varied, resulting in altered lipid compositions of the cells. Lowering the amounts of cholesterol in the media resulted in less cholesterol incorporation into the cell lipid, with a concomitant decrease in the amount of phospholipid. The changes in the phospholipid/cholesterol ratio in the mycoplasmas were very small compared with the large changes in the amount of cholesterol which occurred when the cholesterol content in the medium was altered. Changes in the amounts of glycolipid and glyceride in the cell lipid also resulted from alterations of the cholesterol concentration in the media. Under these conditions cells with reduced cholesterol contents were more sensitive to lysis by digitonin. No changes were observed in the polyacrylamide gel electrophoretic pattern of cellular or membrane proteins when the cholesterol was replaced by other sterols.

INTRODUCTION

The use of a defined growth medium is an essential requirement for any controlled modification of the chemical composition of a micro-organism. Mycoplasmas are only capable of limited metabolism of the lipid components of their cell membranes and are dependent on preformed lipid precursors such as fatty acids and sterols for good growth. By controlling the composition of a growth medium defined for fatty acids and sterol, the lipid composition of mycoplasmas grown in that medium may be modified.

No modification of cholesterol that was incorporated from the growth medium was observed in *Mycoplasma* strains v5 and Y (Rodwell, 1963) or in *Mycoplasma* PG3 among other species (Argaman & Razin, 1965). Fatty acids were not modified in *Mycoplasma* strain Y (Rodwell & Peterson, 1971) but were in *Acholeplasma laidlawii* strain B (McElhaney & Tourtellotte, 1970).

Rodwell (1969) described a partially defined medium, C2, containing an uncharacterized component, 'fraction C', a protein component derived from bovine serum, that acted as a sterol carrier. The fatty acids in the medium are bound to bovine serum albumin. The Edward medium (Razin, 1963) is another medium which, although it is much less well defined than the C2 medium, is often used for growth of mycoplasmas. This paper is concerned with the growth and chemical characterization of *Mycoplasma mycoides* subsp. *capri* (*Mycoplasma* PG3) under a variety of culture conditions in these and other media.

METHODS

Organism. The organism used in this study was *Mycoplasma mycoides* subsp. *capri* (NCT10137), obtained from the Mycoplasma Reference Laboratory, Central Public Health Laboratory, London, N.W. 9.

Maintenance and estimation of growth. Freeze-dried cultures were stored at -20°C . All growth was at 37°C , estimated by the E_{420} using a Zeiss spectrophotometer model PMQ11. Purity of cultures was examined by growth on Hayflick's agar (Chanock, Hayflick & Barile, 1962) in the presence of antiserum discs obtained from Dr R. H. Leach at the Mycoplasma Reference Laboratory, and by microscopic examination.

Growth media. Hayflick's medium was used as described by Chanock *et al.* (1962). Rodwell's (1969) C2 medium was used except for the following alterations to concentrations: adenine, guanine and uracil, 20 mg/l; thymine, 10 mg/l; charcoal-treated bovine serum albumin, 1.0 g/l; pronase digest of fraction C, 0.2 g/l; sodium oleate and sodium palmitate, 0.025 mM each; cholesterol or other sterols, between 0.01 and 0.08 mM according to experimental requirements.

In a variation of the C2 medium, a crude mannan preparation (Thompson, Knights & Parks, 1973) at 1 g/l replaced fraction C. This medium has been designated Cm. Modified Edward medium (Razin, 1963) was used, but with 1 mg fatty acid-free BSA (Sigma)/ml plus 0.025 mM sodium oleate, 0.025 mM sodium palmitate, and cholesterol between 0.01 and 0.08 mM. Harvesting of cells at late exponential phase of growth was performed by centrifugation on an MSE 18 high speed centrifuge for 15 min at 15000 g, and the organisms washed five times in 0.02 M-tris-HCl pH 7.0, containing 0.01 M-MgCl₂·6H₂O.

Assessment of osmotic fragility and sensitivity to digitonin. Osmotic fragility was determined by adding a washed suspension of mycoplasma to either distilled water or solutions of varying tonicity at the required temperature and recording the decrease with time in extinction at 420 nm. Sensitivity to digitonin (up to 50 µg/ml) was determined by suspending washed mycoplasma in a solution of digitonin containing 0.01 M-MgCl₂ and proceeding as with the measurement of osmotic fragility.

Preparation of membranes. Membranes were prepared either by digitonin treatment in the presence of 0.01 M-MgCl₂ (Rottem & Razin, 1972) or by ultrasonic treatment. A Mullard sonicator operating at about 600 W for periods totalling 15 min resulted in about 90 % cell breakage. The membranes were separated from unbroken cells by centrifugation through sucrose density gradients.

Polyacrylamide gel electrophoresis of cell and membrane proteins. The protein-solubilization procedure was as described by Fairbanks, Steck & Wallach (1971). Samples of 100 µg protein were separated on 10 or 12.5 % cross-linked gels containing 0.1 % (w/v) SDS with tris-glycine buffer containing 0.1 % (w/v) SDS at 3 mA/tube. The Fairbanks staining procedure was used, destaining in 10 % (v/v) acetic acid at 37°C . The protein band patterns were recorded on a Joyce automatic recording microdensitometer model MKIIC. Standard protein markers were also run as molecular weight indicators.

Extraction of lipids. Lipids were extracted from both intact cells and membrane preparations. The cell and membrane pellets formed after centrifugation were resuspended in a minimum volume of methanol before the addition of approximately twenty volumes of chloroform-methanol (2:1, v/v). Two successive extractions were made with chloroform-methanol (2:1, v/v), the first at room temperature for 6 h and the second at room temperature overnight. The extracts were pooled, washed twice with water and four times with

chloroform-methanol-water (3:48:47, by vol.) (Longley, Rose & Knights, 1968). The solvent was removed by evaporation under N_2 .

Fractionation and qualitative analyses of neutral and polar lipids. The neutral and polar lipids were separated by silicic acid chromatography (Russell, 1974). Qualitative analysis of these lipids was performed by thin-layer chromatography. Microscope slides, coated with a 0.25 mm layer of Anasil G, were activated just before use by heating at 100 °C for 1 h. The neutral lipids were developed in petroleum ether (b.p. 40 to 60 °C)-diethyl ether-acetic acid (80:20:2, by vol.) and the polar lipids with chloroform-methanol-acetic acid-water (25:15:4:2, by vol.) (Longley *et al.* 1968). The lipid components were visualized by iodine absorption or by charring, and characterized by group specific sprays (Waldi, 1965) and also by co-chromatography with authentic standards.

Quantitative analyses. Protein was estimated using Folin's reagent (Lowry *et al.* 1951). Hexose was estimated by the anthrone procedure using a glucose standard (Trevelyan & Harrison, 1952). Glycolipid was estimated from the hexose analysis of the whole lipid extract, assuming a glycolipid structure such as that found in *Acholeplasma laidlawii* (Shaw, Smith & Koostra, 1968). RNA was estimated by the orcinol method (Herbert, Phipps & Strange, 1971) and DNA by a modified Burton technique (Giles & Myers, 1965). Total phosphorus was assayed by the method of Chen, Toribara & Warner (1956). The phospholipid contents were calculated from the phosphate contents of lipid extracts by the method of Hunter & Rose (1972). Glycerides were estimated according to the method described by Van Handel & Zilversmit (1957) and free fatty acids by the method of Heinen & De Vries (1966). Ergosterol was assayed according to Breivik & Owades (1957). Cholesterol up to 50 µg was estimated by the $FeCl_3$ method (MacIntyre & Ralston, 1954), but for amounts of cholesterol between 1 and 5 µg a modification of the method of Rudel & Morris (1973) was used: the lipid sample was dissolved in chloroform and transferred to a small tube, after which the solvent was removed by evaporation under nitrogen. Then 0.03 ml 33 % (w/v) KOH and 0.3 ml 96 % ethanol were added and mixed. This mixture was heated at 60 °C for 15 min, cooled, and 0.5 ml water was added. Hexane (0.5 ml) was added and, after mixing, the hexane layer was removed. This was repeated three times, the hexane layers were pooled and the hexane removed by evaporation under nitrogen. Then 0.5 ml of the *O*-phthalaldehyde reagent (Zlatkis & Zak, 1969) was added and mixed. After 10 min, the E_{550} was measured using minicells in a Zeiss spectrophotometer. An E_{550} of 0.26 corresponded to 1 µg cholesterol.

Gas-liquid chromatography. Fatty acids and sterols were analysed by g.l.c. using a Pye series 104 chromatograph equipped with dual-flame ionization detectors. Lipid samples were refluxed with methanolic KOH (3 M) for 2 h to saponify fatty acid esters. Free sterols were extracted into heptane and the mixture acidified with HCl for the extraction of fatty acids by heptane. Before analysis by g.l.c. the fatty acids were transmethyalted (Nichols & Moorhouse, 1969).

The fatty acid analyses were performed on a 5 ft column of 10 % (w/w) polyethylene glycol adipate (PEGA) on 100 to 200 mesh celite. Argon flow was 40 ml/min and the column temperature was 190 °C.

The sterol analyses were performed on a 9 ft column of 3.5 % (w/w) polydimethylsiloxane (JXR) on 100 to 200 mesh gas chrome Q. Argon flow was at 60 ml/min and the column temperature was 240 °C.

Peaks were identified by comparison of their retention times with those of authentic standards, and in the case of the fatty acids by comparing retention times on a James plot (James & Martin, 1952).

Table 1. *The chemical compositions of cells and membranes grown in C2 medium*

The medium contained 0.08 mM-cholesterol, 0.025 mM-sodium oleate and 0.025 mM-sodium oleate as the only lipid components.

	Dry wt (%)	
	Cells	Digitonin membranes
DNA	11.4	0
RNA	22.8	7.3
Protein	34.4	37.9
Lipid	24.2	47.0
Hexose	4.7	11.4
Total	97.5	103.6

Materials. Organic solvents were redistilled once before use. Fatty acid standards were obtained from the Hormel Institute, Austin, Minnesota, U.S.A., and shown to be up to 99 % pure by g.l.c. analysis. Cholesterol was obtained from Hopkin and Williams. All other sterols were obtained from Sigma chemicals and with the exception of β -sitosterol (60 % pure) were shown to be pure by g.l.c. analysis.

O-phthalaldehyde was obtained from BDH. All other chemicals were Analar grade whenever possible.

RESULTS

Hayflick's, C2 and Edward media all supported good growth of the organism with doubling times of about 4 to 5 h and a maximum yield of 0.5 mg dry wt mycoplasma/ml culture. No growth occurred in the absence of sterol or of palmitic or oleic acid. *Mycoplasma* PG3 can adapt to growth in very low concentrations of cholesterol (Rottem *et al.* 1973) but for our present work concentrations lower than 0.01 mM were not used. The cholesterol could be replaced by 5 α -cholestan-3 β -ol, ergosterol or β -sitosterol but not by 5 β -cholestan-3 α -ol. This may reflect the requirement for a 3 β hydroxyl group for incorporation of a sterol into a membrane (De Kruffy *et al.* 1973). Growth in Cm was not as good as in C2 although Cm may be used in preference to C2 because of the difficulty in preparing fraction C. A doubling time of 8 h with a maximum yield of 0.2 mg dry wt/ml was obtained when *Mycoplasma* PG3 was grown in Cm.

Chemical characterization

The gross chemical compositions of cells and digitonin-prepared membranes are given in Table 1. The apparent RNA contents may be high because the orcinol in the assay mixture may react with the galactan present in the perchloric acid extract. Indeed, Buttery & Plackett (1960) claimed to have identified small amounts of ribose in the galactan of *M. mycoides* var. *mycoides*. The high hexose content of the membranes will reflect the presence of digitonin in the membrane.

In the C2, Cm and Edward media the only sterols present in the membranes were those which had been added to the media. The sterol and fatty acid contents of mycoplasma grown in Hayflick's medium were complex, reflecting the complexity of the medium (Table 2). Of the fatty acid contents of cells grown in the other media, more than 98 % of the fatty acids were derived without modification from those added to C2 and Cm, compared with more than 90 % in the Edward medium. The fatty acid compositions of cells grown in the Edward medium containing different concentrations of cholesterol or ergosterol are shown in Tables 3 and 4,

Table 2. *The fatty-acid composition of Mycoplasma PG3 grown in Hayflick's medium*

Fatty acid methyl esters were analysed by gas-liquid chromatography, the species recognized by constructing James' plots of the fatty acids before and after hydrogenation (James & Martin, 1952) and by co-chromatography with authentic standards.

Fatty acid	Composition (%)	Fatty acid	Composition (%)
*	4.16	16:1	7.54
12:0	3.05	17:0	2.20
*	4.00	17:1	2.70
14:0	3.23	18:0	9.35
*	4.40	18:1	12.72
15:0	3.91	19:0	3.69
15:1	2.75	19:1	1.10
16:0	35.50		

* An unidentified fatty acid.

Table 3. *The fatty acid composition of Mycoplasma PG3 grown in different concentrations of cholesterol in Edward medium*

Fatty acid	Composition (%)		
	0.8 C*	0.4 C*	0.1 C*
12:1	1.15	1.80	1.93
14:0	4.82	3.12	2.36
16:0	56.40	58.30	62.00
16:1	3.53	3.44	2.36
18:0	1.44	1.35	1.30
18:1	32.80	31.80	30.20
16:0 to 18:1 ratio	1.72	1.84	2.05

* 0.8 C, 0.4 C and 0.1 C represent 0.08, 0.04 and 0.01 mM-cholesterol in the medium, respectively.

Table 4. *The fatty acid composition of Mycoplasma PG3 grown in different concentrations of ergosterol in Edward medium*

Fatty acid	Composition (%)		
	0.8 E*	0.4 E*	0.1 E*
12:1	0.89	0.93	0.23
14:0	1.99	2.21	1.15
16:0	54.40	57.10	62.40
16:1	3.88	3.22	2.59
18:0	2.00	2.24	1.96
18:1	34.00	31.10	28.90
19:0	2.88	2.42	2.82
16:0 to 18:1 ratio	1.60	1.84	2.16

* 0.8 E, 0.4 E and 0.1 E represent 0.08, 0.04 and 0.01 mM-ergosterol in the medium, respectively.

respectively. Unlike the values reported by Rottem *et al.* (1973) for cholesterol-grown cells, these values indicate that there is very little $C_{18:0}$ fatty acid present when none is added to the medium and that the medium is virtually free from endogenous fatty acids.

The composition of lipid extracted from cells grown in C2 medium containing different cholesterol contents is given in Table 5. The only phospholipids were cardiolipin, phosphatidyl glycerol and phosphatidic acid. The amount of cholesterol in C2, Cm and the Edward medium was altered to study the regulation of the phospholipid:cholesterol ratio

Table 5. *Analyses of lipids extracted from Mycoplasma PG3 grown with different cholesterol concentrations in C2 medium*

Lipid component	Total lipids (% w/w)		
	0.8 C*	0.4 C*	0.1 C*
Cholesterol	18.1	14.1	5.2
Total phospholipid	64.0	54.0	21.1
Glycolipid†	10	12	18
Free fatty acid	5	5	5
Triglyceride	3.0	5.0	12.1
Mono- and di-glyceride	8.5	12.5	26.5
Total	107.6	103.6	97.9

* For nomenclature see Table 3.

† Approximate values based on hexose estimation of total lipid (see Methods).

Table 6. *The cholesterol and total phospholipid contents, as a percentage of total extracted lipid, of Mycoplasma PG3 grown in three different media, each with different concentrations of cholesterol*

	0.8 C*			0.4 C*			0.1 C*		
	C2	Cm	Edward	C2	Cm	Edward	C2	Cm	Edward
Cholesterol	18.1	19.4	18.4	14.1	14.6	14.2	5.2	5.0	9.5
Phospholipid	64	56	52	54	40	48	21	17	30
Molar ratio of phospholipid to cholesterol	1.73	1.40	1.37	1.86	1.34	1.64	1.90	1.76	1.54

* For nomenclature see Table 3.

(Table 6). The range of phospholipid:cholesterol ratios, 1.34 to 1.90, is considered small in comparison with the three- to fourfold change in the cholesterol contents of the cell lipid.

Osmotic and digitonin sensitivity

Osmotic lysis of washed suspensions of *Mycoplasma* PG3 did not occur to any great extent no matter what growth conditions were used. Suspension in water at 37 °C resulted in about 20 % lysis of the cells after 30 min, whereas at 0 °C no appreciable lysis occurred. The sensitivity to digitonin of C2-grown cells, grown in three different concentrations of cholesterol at 20 °C, is shown in Fig. 1. The sensitivity at 37 °C was not significantly greater than at 20 °C, a result in agreement with those of Rottem & Razin (1972). No lysis was observed at 0 °C. For a given concentration of digitonin, cells with a lower amount of cholesterol in their membranes were lysed to a greater extent.

Polyacrylamide gel electrophoresis

Figure 2 shows the electrophoretic patterns of the proteins from whole-cell and membrane proteins run on 10 and 12½ % cross-linked gels respectively. These patterns were all obtained when cholesterol was the only sterol present in the growth medium. When the cholesterol was replaced by ergosterol, β -sitosterol or 5 α -cholestan-3 β -ol, no differences in the protein band patterns were observed.

Fig. 1. The sensitivity of *Mycoplasma* PG3 to digitonin. The cells were grown in C2 medium containing different concentrations of cholesterol and then washed and resuspended at 20 °C in buffer containing digitonin. Cells were either suspended in 25 µg digitonin/ml after growth in (○) 0.08 mM, (●) 0.04 mM, or (△) 0.01 mM-cholesterol, or they were suspended in 50 µg digitonin/ml after growth in (▲) 0.08 mM, (□) 0.04 mM, or (■) 0.01 mM-cholesterol.

Fig. 2. Polyacrylamide gel electrophoretograms of whole cell and membrane proteins. (a) Whole cell protein, and (b) membrane protein, on 10 % cross-linked gel; (c) whole cell protein, and (d) membrane protein, on 12.5 % cross-linked gel.

DISCUSSION

When the cholesterol content of the cell membranes is varied by altering the concentration of cholesterol in the growth medium, it is apparent that both the cholesterol and phospholipid contents of the extracted lipids are controlled, since, in all experiments reported here, the phospholipid/cholesterol molar ratio was maintained between one and two. Healthy red blood cells which also have limited metabolic capabilities maintain such a ratio above unity although red blood cells from certain unhealthy subjects may show a much higher proportion of cholesterol (Vanderkooi *et al.* 1974). The metabolically-inactive myelin membrane has a phospholipid:cholesterol ratio of one (O'Brien & Sampson, 1965). In artificial membranes, the sterol-phospholipid interaction has been extensively studied, a 3 β -hydroxyl group being required on the sterol (De Kruffy *et al.* 1973) located next to phosphate in cholesterol-lecithin vesicles (Phillips & Finer, 1974). The phospholipid:cholesterol ratio in artificial membranes influences the lipid fluidity (Ladbrooke, Williams & Chapman, 1968) and controls many membrane properties such as ion permeability (De Gier, Mandersloot & Van Deenen, 1968; Papahadjopoulos, 1970). The living cell can adapt to varying growth conditions by an alteration in the phospholipid and cholesterol content of its membrane, although other lipid components may well be important. The increase in the diglyceride content of cholesterol-depleted cells confirms the suggestion of Rottem *et al.* (1973) that this is so. The diglyceride and monoglyceride spots on the thin-layer chromatograms could not be separated sufficiently for independent quantitative analyses to be performed. In *Acholeplasma laidlawii*, glycolipids constitute about half of the total lipids, this proportion being unaffected by the concentration of cholesterol in the medium (Shaw *et al.* 1968). *Acholeplasmas*, unlike *mycoplasmas*, are not obligate sterol requirers, and so the increase in glycolipid content in *Mycoplasma* PG3 when the cholesterol content is reduced may not be contradictory to the result obtained with *A. laidlawii*. In these studies, the only fatty acids present in the medium were oleic and palmitic. When the cholesterol content of the cells was reduced, the ratio of the saturated to unsaturated fatty acids in the cells increased, contributing to the maintenance of lipid fluidity in the membrane, a result in agreement with that reported by Rottem *et al.* (1973). The precipitation of cholesterol by digitonin is well known (Moore & Baumann, 1952) and digitonin has been used to differentiate between *Mycoplasmas* and *Acholeplasmas* (Freundt *et al.* 1973). Cholesterol confers digitonin sensitivity on bacterial L-phase variants (Razin & Shafer, 1969), and to lecithin liposomes (Hsu Chen & Feingold, 1973). That the *mycoplasmas* I used, grown under conditions such that their membrane cholesterol content was reduced, were repeatedly more sensitive to lysis by digitonin, must be attributed to the overall state of organization in the membrane rather than the cholesterol content alone.

The polyacrylamide gel electrophoretograms of cell and membrane proteins show that few differences exist. When densitometer traces of these gels are compared it can be seen that three proteins are missing from the membrane protein gel, in the high molecular weight region, and that four others are present in greatly reduced amounts compared with the whole-cell protein gel. No significant differences were apparent when the cholesterol was substituted by ergosterol, β -sitosterol or 5 α -cholestan-3 β -ol, although any differences may well be very limited particularly in view of the small differences between the whole-cell protein and membrane protein profiles. Pisetsky & Terry (1972), looking at the effect of fatty acid composition on the membrane proteins of *A. laidlawii*, suggested that although the same proteins were always present quantitative differences did exist when the fatty acid composition was altered.

I am grateful to Dr D. Kerridge for his interest and helpful suggestions and to Professor E. F. Gale and Dr P. Whittlestone for reading the manuscript. This work was performed during the tenure of a Medical Research Council Award.

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