The Effect of Growth Temperature on Wax Ester Composition in the Psychrophilic Bacterium *Micrococcus cryophilus* ATCC 15174

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The wax ester and constituent fatty alcohol and fatty acid compositions of the psychrophilic bacterium *Micrococcus cryophilus* grown at 1 or 20 °C have been analysed using packed column and capillary gas-liquid chromatography. The major wax esters were C₃₆, C₃₄ and C₃₂, with a large percentage of mono- and di-unsaturated wax esters (91 to 99 % of the total). When the growth temperature was lowered from 20 to 1 °C the average chain length of the wax esters decreased and their unsaturation increased. The average chain length of the saturated wax esters was less than that of the unsaturated wax esters. The fatty alcohols and fatty acids of the wax esters were mainly C₁₈, with smaller amounts of C₁₆, and were straight chain saturated and mono-unsaturated with *cis* double bonds at positions Δ9 and Δ11. The fatty alcohols had a shorter average chain length and were more saturated than the fatty acids, and contained a higher proportion of *cis* Δ11 unsaturated isomers. The combination of fatty alcohols and fatty acids in wax esters appeared to be random. The significance of these results is discussed in relation to the taxonomy of *M. cryophilus*, growth temperature-dependent membrane fluidity changes and the biosynthesis of wax esters.

INTRODUCTION

Wax esters are found throughout the plant and animal kingdoms (Kolattukudy, 1976) and in a few species of bacteria (Bryn *et al.*, 1977; Albro, 1976), commonly in *Acinetobacter* species (Gallagher, 1971; Thorne *et al.*, 1973; Fixter & Fewson, 1974; Makula *et al.*, 1975; Bryn *et al.*, 1977). The presence of wax esters in Gram-negative cocci and their degree of unsaturation have been used in the classification of this group of bacteria (Bryn *et al.*, 1977). However, it is not clear whether bacterial waxes are components of membranes. Wax esters have been detected in outer membrane fractions of *Acinetobacter* strain MJT/F5/199A (Thorne *et al.*, 1973) and in the membranes (combined inner plus outer) of *Micrococcus cryophilus* (Russell, 1978*a*). It could be argued that the association with membranes is an artifact of isolation, although in *M. cryophilus* the wax ester composition is modified by changes in growth temperature (Russell, 1978*a*), which is typical of membrane lipids. On the basis of the results of experiments in which the wax ester content of *Acinetobacter calcoaceticus* grown in a chemostat under conditions of nitrogen or phosphorus limitation were studied, Fixter & Fewson (1974) concluded that in this micro-organism the wax esters may be used as energy stores. There are too few studies, however, to draw any general conclusions about the function of wax esters in bacteria.

Our interest in bacterial wax esters is twofold. Firstly, wax esters have been found in aquatic sediments (Boon & de Leeuw, 1979) and a knowledge of the acyl composition (e.g.

chain length, position and geometry of double bonds) should be helpful in determining the origin of these lipids in such sediments. There is a paucity of information, however, on the structure of bacterial wax esters. Secondly, a detailed study of wax ester composition would provide important information as a preliminary to the study of the mechanism of biosynthesis of wax esters in bacteria. Although the synthesis of fatty alcohols has been studied in *Escherichia coli* (Naccarato *et al.*, 1972; 1974), *Mycobacterium tuberculosis* (Wang *et al.*, 1972) and *Clostridium butyricum* (Day *et al.*, 1970), the complete synthesis of wax esters has not been studied in any bacterial species (Albro, 1976).

The present paper reports a detailed study of the wax ester composition of the psychrophilic bacterium *Micrococcus cryophilus* ATCC 15174; this is a Gram-negative coccus which resembles *Acinetobacter* species and contains a high proportion of wax esters in its lipid (Russell, 1974). The use of capillary gas-liquid chromatography has revealed the presence of positional isomers of unsaturated fatty alcohols and acids. The implications of this finding for the mechanism of their biosynthesis are discussed.

METHODS

Materials. Wax esters, fatty alcohols and fatty acids were obtained from Sigma or Nu-Chek Prep., Elysian, Mn. 56028, U.S.A. Reagents used were of the highest purity obtainable.

Maintenance and growth of cultures. *Micrococcus cryophilus* ATCC 15174 (the type strain) was grown at 1 or 20 °C in a Casamino acids/salts medium (NM medium) (Russell, 1974) for at least two subcultures and four doubling times at the appropriate growth temperature prior to harvesting the bacteria for lipid extraction and analysis. The organism was maintained at 4 °C on NM medium containing 2% (w/v) agar (Russell, 1974).

Lipid extraction and analysis. Bacteria were harvested in late-exponential phase and the total lipid was extracted with chloroform/methanol mixtures (see Russell, 1974). Wax esters were isolated from total lipid extracts by preparative thin-layer chromatography (t.l.c.) on 0.3 mm silica gel H (Merck) plates using light petroleum (b.p. 60 to 80 °C)/diethyl ether/acetic acid (85:15:1, by vol.) as developing solvent. The wax ester band (R_F about 0.85) was visualized with Rhodamine 6G (0.012%, w/v), scraped off and the wax esters were eluted with three washes of diethyl ether. When required, wax fatty alcohols and wax fatty acids were prepared by saponification of the wax esters using 3 M-KOH in methanol. After dilution with water and acidification the products were extracted into light petroleum (b.p. 30 to 40 °C) and treated with diazomethane. The resulting wax fatty alcohols and wax fatty acids (as methyl esters) were separated by preparative t.l.c., as above, using light petroleum (b.p. 60 to 80 °C)/diethyl ether (8:2, v/v) as developing solvent. Alternatively, wax fatty alcohols were extracted under alkaline conditions from saponification reaction mixtures, which were then acidified and wax fatty acids were extracted and esterified with diazomethane or BF_3 /methanol (14%, w/w). In some experiments, wax esters were fractionated into saturated (R_F 0.73), mono-unsaturated (R_F 0.49) and di-unsaturated (R_F 0.36) wax esters by preparative argentation t.l.c. on 0.3 mm silica gel HF₂₅₄₊₃₆₆ (type 60) plates containing AgNO_3 (12.5%, w/w) using hexane/diethyl ether (9:1, v/v) as developing solvent.

Gas-liquid chromatography (g.l.c.). Wax esters were analysed directly with a Carlo Erba FTV 2151 gas chromatograph fitted with 12 m $\times$ 0.3 mm (i.d.) or 20 m $\times$ 0.3 mm (i.d.) wall-coated open tubular (WCOT) glass capillary columns coated with OV-1 according to the method of Grob *et al.* (1978). The use of the shorter column verified the absence of wax esters of chain length greater than C_{38} . The carrier gas was helium at an inlet pressure of 0.9 kgf cm⁻² (12 m column) or 1.4 kgf cm⁻² (20 m column) corresponding to a flow of about 1.5 ml min⁻¹. The oven temperature was programmed from 100 to 280 °C at 4 °C min⁻¹ and held isothermally at 280 °C for 30 min to elute the C_{38} wax esters. A flame ionization detector was employed with injector and detector temperatures of 300 °C. Peak areas were measured manually by triangulation (peak height $\times$ peak width at half height) and by electronic integration using an Infratronics 308 computing integrator. All calculations were performed at least in triplicate and the average was used to calculate the results presented in Table 1. Equal flame ionization detector response factors were assumed for all the wax esters (Bryn *et al.*, 1977).

Fatty acids (as methyl esters) and fatty alcohols (as acetates) derived from saponification of the wax esters were analysed by packed column g.l.c. using a Perkin-Elmer F33 gas chromatograph fitted with a flame ionization detector. The 1.5 m $\times$ 5 mm (i.d.) glass columns were packed with 15% (w/w) ethylene glycol succinate silicone (EGSS-X) on 80 to 100 mesh Chromosorb W AW (Supelco, Bellefont, Pa. 16823, U.S.A.).

Separations were achieved isothermally at 190 °C with detector and injector temperatures of 225 °C. Peak areas were measured using a Varian CDS III integrator, and agreed to within 2 % with values obtained using capillary g.l.c.

Fatty acid methyl esters and fatty alcohols, both as trimethylsilyl ethers and acetate derivatives, were analysed on the 20 m OV-1 glass capillary column, as described for the wax ester samples (see above), except that a lower inlet pressure of 0.7 kgf cm⁻² was used giving a flow of about 1 ml min⁻¹. This column provided almost complete resolution for each pair of double-bond positional isomers and these could be co-injected with available authentic standards. Additional data on the double bond positions in the fatty acid methyl esters were obtained using a 100 m × 0.3 mm (i.d.) WCOT glass capillary column coated with diethylene glycol succinate, operated isothermally at 170 °C with helium as the carrier gas. This capillary g.l.c. column provided a useful check on the identifications made, since the order of elution of the saturated and mono-unsaturated fatty acid methyl esters is the reverse of that on the apolar OV-1 columns.

Identification of double bond position by mild permanganate oxidation. The position of the double bond in the fatty acids and fatty alcohols deduced from co-injection on the capillary g.l.c. columns was confirmed using the KMnO₄ oxidation procedure of Downing & Greene (1968), as described previously (Russell, 1978*b*). Free fatty alcohols were oxidized efficiently by this procedure to produce dicarboxylic acid fragments by oxidation of the primary alcohol group to a carboxyl group, in addition to the oxidative cleavage of the double bond.

Infrared spectroscopy. The presence of *trans* unsaturated isomers was checked using infrared spectroscopy. Wax ester, fatty acid and fatty alcohol samples were spread as a thick film between the plates of NaCl cells and spectra were recorded using a Perkin-Elmer model 137B Infracord spectrometer operated at room temperature without any reference cell.

Combined capillary g.l.c./mass spectrometry. Mass spectra of the fatty acid methyl esters, fatty alcohols (as trimethylsilyl ethers and acetates) and wax esters were obtained using a Finnigan 4000 combined capillary g.l.c./quadrupole mass spectrometer linked to a laboratory PDP-8e computer system. Mass spectra were taken every 2.2 s at an electron energy of 40 eV, electron current of 350 µA, accelerating voltage of 2 kV and ion source temperature of 250 °C. A 20 m × 0.3 mm (i.d.) glass capillary WCOT OV-1 column coupled directly to the ion source using glass-lined stainless steel tubing was used. The g.l.c. conditions were similar to those described above. The mass spectra obtained were compared with those of available standards and with those in the literature (Aasen *et al.*, 1971; Spencer, 1979).

Melting point determinations. For each available wax ester standard (Sigma) at least three melting point determinations were made using capillary tubes; individual measurements agreed to within 1 °C.

RESULTS

Wax ester composition

The results of analyses of intact wax ester samples extracted from *Micrococcus cryophilus* grown at 1 or 20 °C are summarized in Table 1. The predominant wax esters in all samples were C₃₆, C₃₄ and C₃₂, in order of decreasing abundance, with a large proportion being unsaturated. The degree of unsaturation of the wax esters increased as the growth temperature decreased. Bacteria grown at 20 °C contained a higher proportion of saturated and mono-unsaturated wax esters and a lower proportion of di-unsaturated wax esters compared with bacteria grown at 1 °C, which contained < 1 % saturated wax esters. These differences were reflected in an increase in the wax ester unsaturation index [i.e. (% mono-unsaturated + 2 × % di-unsaturated) / 100] from 1.41 for bacteria grown at 20 °C to 1.84 for bacteria grown at 1 °C. These growth temperature-dependent changes in unsaturation did not appear to be associated with wax esters of a particular chain length. However, if the chain length of wax esters of bacteria grown at a particular temperature were studied, it was noticeable that the average chain length (as exemplified by the C₃₆/C₃₄ and C₃₆/C₃₂ ratios—see Table 1) was greater for the di-unsaturated than for the mono-unsaturated wax esters, which in turn had a greater average chain length than the saturated wax esters. The overall picture was complicated by the concomitant growth temperature-dependent chain length changes, the average chain length of all wax ester classes decreasing with a reduction in growth temperature. When the total wax esters were considered, the C₃₆/C₃₄ and C₃₆/C₃₂ ratios, calculated from the results in Table 1, decreased from 2.60 and 16.90 in bacteria grown at 20 °C to 1.57 and 7.92 in bacteria grown at 1 °C.

Table 1. *Percentage composition of wax esters in Micrococcus cryophilus grown at 1 or 20 °C*

Wax esters were analysed by capillary g.l.c. as described in Methods, and amounts of wax esters are given as the mean areas of individual peaks ($\pm$ standard deviation) expressed as a percentage of the total area under the chromatogram. The number of cultures extracted is given by *n*. At least three analyses were performed on the wax esters of each culture. Wax esters are designated by 'number of carbon atoms:number of double bonds'. A and B refer to the major peaks of each mono- and di-unsaturated wax ester of a particular chain length (see text and Fig. 1). 36/34 and 36/32 are the ratios of the percentages of wax esters of the chain lengths indicated within a class (saturated, mono-unsaturated, di-unsaturated).

Wax ester	Growth at 1 °C (<i>n</i> = 2)			Growth at 20 °C (<i>n</i> = 3)		
	Composition (%)	36/34	36/32	Composition (%)	36/34	36/32
Saturated						
30:0	tr			0.3 $\pm$ 0.1		
32:0	0.3 $\pm$ 0.4			1.2 $\pm$ 0.4		
34:0	0.4 $\pm$ 0.1			3.6 $\pm$ 0.9		
36:0	0.2 $\pm$ 0.0			3.2 $\pm$ 1.1		
38:0	tr			0.3 $\pm$ 0.3		
Others	tr			0.4 $\pm$ 0.3		
Total saturated	0.9	0.50	0.67	9.0	0.89	2.67
Mono-unsaturated						
30:1 Δ 9 (A)	0.2 $\pm$ 0.1			0.5 $\pm$ 0.3		
30:1 Δ 11 (B)	0.2 $\pm$ 0.1			0.1 $\pm$ 0.0		
32:1 Δ 9 (A)	1.4 $\pm$ 0.9			1.9 $\pm$ 0.5		
32:1 Δ 11 (B)	1.5 $\pm$ 0.9			0.4 $\pm$ 0.1		
34:1 Δ 9 (A)	8.7 $\pm$ 6.9			13.8 $\pm$ 0.4		
34:1 Δ 11 (B)	0.6 $\pm$ 0.6			2.7 $\pm$ 0.6		
36:1 Δ 9 (A)	2.5 $\pm$ 3.0			24.6 $\pm$ 3.0		
36:1 Δ 11 (B)	0.1 $\pm$ 0.1			4.2 $\pm$ 2.6		
38:1 Δ 9 (A)	tr			0.9 $\pm$ 0.1		
38:1 Δ 11 (B)	tr			0.2 $\pm$ 0.0		
Others	0.3 $\pm$ 0.2			1.0 $\pm$ 0.1		
Total mono-unsaturated	15.5	0.28	0.90	50.3	1.75	12.52
Di-unsaturated						
32:2 (A)	2.3 $\pm$ 0.6			0.2 $\pm$ 0.0		
32:2 (B)	1.6 $\pm$ 0.4			0.2 $\pm$ 0.0		
34:2 (A)	9.6 $\pm$ 1.5			2.8 $\pm$ 0.3		
34:2 (B)	16.4 $\pm$ 1.5			2.5 $\pm$ 0.7		
36:2 (A)	41.6 $\pm$ 6.4			18.1 $\pm$ 2.5		
36:2 (B)	11.8 $\pm$ 4.5			15.8 $\pm$ 2.9		
38:2 (A)	tr			0.5 $\pm$ 0.2		
38:2 (B)	tr			0.3 $\pm$ 0.0		
Others	0.7 $\pm$ 0.1			0.6 $\pm$ 0.0		
Total di-unsaturated	84.0	2.05	13.69	41.0	6.40	84.75

tr, Trace (less than 0.1 %).

Previous analyses of bacterial and other wax esters (e.g. Tulloch, 1975; Tagaki *et al.*, 1976; Bryn *et al.*, 1977) have employed conventional packed g.l.c. columns, which provide relatively poor separation of wax esters differing in their degree of unsaturation. Such wax esters were well separated by the capillary columns used here. In addition, the mono-unsaturated wax esters were resolved into two components (Fig. 1), A and B, due to the presence of both *cis* Δ 9 and *cis* Δ 11 isomers (see below). Compounds A are *cis* Δ 9 wax esters; the double bond may be in the fatty alcohol or fatty acid, since, for instance, stearyl oleate

Fig. 1. Chromatogram of wax esters of *Micrococcus cryophilus* grown at 20 °C, analysed using the 20 m wall-coated open tubular capillary column (for details, see Methods). Wax esters are designated by 'number of carbon atoms:number of double bonds'. A and B refer to the $\Delta 9$ and $\Delta 11$ isomers, respectively, of mono-unsaturated wax esters, and to the major peaks of di-unsaturated wax esters.

and oleyl stearate co-elute. Compounds B are *cis* $\Delta 11$ wax esters. The di-unsaturated wax esters were resolved into two major peaks (A and B) and one minor peak (Fig. 1). Excluding the minor contributions from C_{14} and C_{20} fatty alcohols and fatty acids to C_{36} and C_{38} wax esters, it is believed that compounds A are $\Delta 9$ – $\Delta 9$ di-unsaturated wax esters [using the shorthand notation of Aasen *et al.* (1971), in which the fatty alcohol precedes the fatty acid], compounds B are a mixture of $\Delta 9$ – $\Delta 11$ and $\Delta 11$ – $\Delta 9$ wax esters, and the minor compounds are $\Delta 11$ – $\Delta 11$ wax esters.

Wax ester fatty alcohol and fatty acid composition

The major fatty alcohols and fatty acids of the wax esters were C_{18} , with smaller amounts of C_{16} (Tables 2 and 3). The fatty alcohols were more saturated than the fatty acids; the ratio of saturated/unsaturated fatty alcohols in wax esters of bacteria grown at 20 °C was 0.94, compared with a value of 0.48 for the fatty acids. The corresponding values of this ratio for fatty alcohols and fatty acids of wax esters in bacteria grown at 1 °C were 0.11 and 0.08, respectively. These values show that when the bacterial growth temperature was lowered the degree of unsaturation of both fatty alcohols and fatty acids increased. In contrast, there was less change in their chain length. The C_{18}/C_{16} ratios of combined saturated and unsaturated fatty alcohols in wax esters of bacteria grown at 20 and 1 °C, calculated from the results in Tables 2 and 3, were 4.13 and 3.45, respectively; the corresponding values for wax ester fatty acids were 4.64 and 4.01. These results also show that the average chain

Table 2. *Percentage composition of fatty alcohols in wax esters of Micrococcus cryophilus grown at 1 or 20 °C*

See legend to Table 1 and Methods for details. Peaks of 12:1, 14:1 and 20:1 were too small a percentage of the total to be resolved accurately into $\Delta 9$ and $\Delta 11$ isomers; the total for each peak is given. The ratios of $\Delta 9$ to $\Delta 11$ isomers in 16:1 and 18:1 are represented by 16:1, $\Delta 9/\Delta 11$ and 18:1, $\Delta 9/\Delta 11$, respectively.

Fatty alcohol	Growth at 1 °C (<i>n</i> = 2)		Growth at 20 °C (<i>n</i> = 3)	
	Composition (%)	18/16	Composition (%)	18/16
Saturated				
12:0	0.1 $\pm$ 0.0		0.5 $\pm$ 0.5	
14:0	0.6 $\pm$ 0.3		1.7 $\pm$ 0.6	
16:0	6.6 $\pm$ 3.0		12.9 $\pm$ 2.5	
18:0	2.9 $\pm$ 2.3		31.7 $\pm$ 7.2	
20:0	tr		1.3 $\pm$ 0.9	
Others	ND		ND	
Total saturated	10.2	0.44	48.1	2.46
Mono-unsaturated				
12:1	ND		ND	
14:1	0.5 $\pm$ 0.2		0.4 $\pm$ 0.2	
16:1 $\Delta 9$	7.7 $\pm$ 1.4		1.7 $\pm$ 0.5	
16:1 $\Delta 11$	7.8 $\pm$ 1.9		3.8 $\pm$ 1.9	
18:1 $\Delta 9$	59.9 $\pm$ 5.9		27.3 $\pm$ 2.7	
18:1 $\Delta 11$	13.4 $\pm$ 6.5		17.0 $\pm$ 4.9	
20:1	0.1 $\pm$ 0.1		1.2 $\pm$ 1.0	
Others	0.5 $\pm$ 0.1		0.4 $\pm$ 0.0	
Total mono-unsaturated	89.9	4.73	51.8	8.06
16:1, $\Delta 9/\Delta 11$	0.99		0.45	
18:1, $\Delta 9/\Delta 11$	4.47		1.61	

tr, Trace; ND, not detected.

length of the wax ester fatty acids was slightly greater than that of the fatty alcohols, at both growth temperatures.

Capillary g.l.c. resolved the mono-unsaturated fatty alcohols and fatty acids into two components, which were identified as *cis* $\Delta 9$ and *cis* $\Delta 11$ isomers on the basis of their co-chromatography with authentic standards on both polar and non-polar column coatings and several different chromatographic conditions. In addition, the isomers of $C_{16:1}$ and $C_{18:1}$ fatty alcohols and fatty acids were identified as *cis*, rather than *trans*, on the basis of their co-chromatography in the *cis*-unsaturated band on argentation t.l.c.; unsaturated isomers were not recovered from the saturated band with which potential *trans* isomers would have co-chromatographed (Morris, 1966). Infrared spectroscopy was also used to demonstrate the absence of *trans* isomers. The exclusion of *trans* isomers using an independent method was believed to be important, because in certain of the gas chromatographic systems used the *cis* $\Delta 11$ isomers co-eluted with *trans* $\Delta 9$ isomers. Typical infrared spectra of the wax esters of *M. cryophilus* and authentic stearyl oleate are shown in Fig. 2. Although di-unsaturated wax ester standards were not available, the spectra of *M. cryophilus* wax esters corresponded closely with those of *cis*-mono-unsaturated wax ester standards. As a check, the spectra of wax ester constituent fatty alcohols and fatty acids were compared directly with oleic acid, oleyl alcohol and elaidic acid standards. The absorption peaks at 2850 to 3000 cm^{-1} , due to methyl and methylene deformation vibrations, are roughly constant amongst the C_{18} compounds used (Jart, 1960). Since the major fatty alcohols and fatty acids in *M. cryophilus* wax esters are C_{18} , the 2850 to 3000 cm^{-1} absorption band could be used as an internal standard. In this way it was possible to record spectra of wax esters (Fig. 2), fatty alcohols and fatty acids under conditions that would have detected the

Table 3. *Percentage composition of fatty acids in wax esters of Micrococcus cryophilus grown at 1 or 20 °C*

See legends to Tables 1 and 2 for details.

Fatty acid	Growth at 1 °C (<i>n</i> = 3)		Growth at 20 °C (<i>n</i> = 3)	
	Composition (%)	18/16	Composition (%)	18/16
Saturated				
12:0	0.5 ± 0.2		0.9 ± 0.4	
14:0	0.7 ± 0.5		1.9 ± 0.3	
16:0	4.8 ± 1.9		13.9 ± 2.0	
18:0	1.9 ± 1.2		15.2 ± 2.0	
20:0	ND		0.4 ± 0.1	
Others	ND		ND	
Total saturated	7.9	0.40	32.3	1.09
Mono-unsaturated				
12:1	ND		ND	
14:1	ND		0.1 ± 0.1	
16:1 Δ9	4.0 ± 1.6		2.0 ± 0.3	
16:1 Δ11	10.8 ± 0.5		1.1 ± 0.5	
18:1 Δ9	72.7 ± 0.7		57.8 ± 2.8	
18:1 Δ11	4.0 ± 2.4		5.8 ± 0.7	
20:1	0.1 ± 0.0		0.3 ± 0.3	
Others	0.5 ± 0.1		0.7 ± 0.2	
Total mono-unsaturated	92.1	5.18	67.8	20.52
16:1, Δ9/Δ11	0.37		1.82	
18:1, Δ9/Δ11	18.18		9.97	

ND, Not detected.

characteristic *trans* absorption at 962 cm⁻¹ (Bellamy, 1958). Such an absorption was never detected in the samples from *M. cryophilus*, indicating the absence of *trans* isomers.

Additional support for the identification of the C_{18:1} fatty alcohol and fatty acid isomers was provided by the determination of the position of the double bond by mild permanganate oxidation. G.l.c. analysis of the methylated dicarboxylic acid fragments of fatty alcohols and fatty acids revealed the presence of C₉ and C₁₁ dicarboxylic acid dimethyl esters in the ratios expected from the intact fatty alcohol and fatty acid capillary g.l.c. analyses. There was not enough material, however, to repeat such an analysis for the C_{16:1} fatty alcohols and fatty acids, so that their designation as Δ9 and Δ11 positional isomers rests upon the capillary g.l.c. analysis only.

The results in Tables 2 and 3 showed that the proportions of Δ9 and Δ11 isomers differed markedly in the C_{16:1} and C_{18:1} components of both fatty alcohols and fatty acids; the Δ9/Δ11 ratio of C_{18:1} was much higher than that of C_{16:1}, irrespective of the growth temperature. The Δ9/Δ11 ratio of the combined C₁₆+C₁₈ fatty acids (5.2 to 8.7:1) was higher than that of the combined C₁₆+C₁₈ fatty alcohols (1.4 to 3.2:1) throughout the growth temperature range. When the growth temperature was raised from 1 to 20 °C there was an increase in the proportion of Δ11 isomers in the C_{18:1} fatty alcohols and fatty acids relative to both C_{18:1}Δ9 and total fatty alcohol or fatty acid contents. It was difficult to draw corresponding conclusions regarding the relative proportions of Δ9 and Δ11 isomers of C_{16:1} in bacteria grown at 20 °C, due to the small amounts of C_{16:1} fatty alcohols and fatty acids produced at this temperature.

Fig. 2. Typical infrared spectra of wax esters of *Micrococcus cryophilus* grown at 20 °C (A) and of stearyl oleate (B). Infrared spectra were obtained using thick films in NaCl cells as detailed in Methods.

Fig. 3. Comparison of the observed and calculated random wax ester profiles of *Micrococcus cryophilus* grown at 20 or 1 °C. The composition of wax esters of *M. cryophilus* as measured by capillary g.l.c. ($\square$) is compared with the wax ester composition calculated by assuming a random combination of the fatty alcohol and fatty acids prepared by saponification of the same wax ester sample ($\boxtimes$). For the purposes of calculating the random composition the values of $\Delta 9$ and $\Delta 11$ isomers have been combined for each particular chain length. To make comparisons easier, results are shown for the major wax ester chain lengths only; these accounted for about 95 % of the total wax esters.

Relationship of fatty alcohol and fatty acid composition to wax ester composition

The fact that the major fatty alcohols and fatty acids were C_{18} , with smaller amounts of C_{16} , together accounted for the pattern of chain lengths of the intact wax esters, in which C_{36} predominated with smaller amounts of C_{34} and C_{32} . The observed wax ester distribution was due, apparently, to a random combination of fatty alcohols and fatty acids, both in terms of the chain length distribution within the saturated, mono-unsaturated and di-unsaturated classes, and the relative amounts of each class (Fig. 3). The largest discrepancy between theoretical and observed frequencies was in the $C_{34:1}$ and $C_{36:1}$ wax esters; otherwise, the values corresponded closely for wax esters of bacteria grown at 1 or 20 °C.

Melting points of mono-unsaturated wax esters

The melting points of three isomers of $C_{36:1}$ wax esters were: for stearyl palmitoleate (18:0–16:1), 29.5 °C; for oleyl palmitate (18:1–16:0), 31 °C; for palmityl oleate (16:0–18:1), 20.5 °C. Unfortunately no authentic sample of palmitoleyl stearate (16:1–18:0) was available.

DISCUSSION

The present paper confirms and extends previously published data on the wax ester fatty alcohol and fatty acid compositions of *Micrococcus cryophilus* obtained using different g.l.c. conditions (Russell, 1978*a*), as well as providing new, detailed information about wax ester compositional changes related to growth temperature. There are only a few examples of the use of capillary g.l.c. to study wax ester composition, and the technique has not been used previously to study bacterial wax esters; the potential of the technique is exemplified by the novel discovery of double bond positional isomers of wax ester unsaturated fatty alcohols and fatty acids.

Wax ester analysis has been used as an aid in the classification of strains of the family *Neisseriaceae* by Bryn *et al.* (1977) who showed that the wax esters of *Acinetobacter* spp. contained a much higher proportion of di-unsaturated wax esters (30 to 98 % of the total) than did 'false neisseriae' and *Moraxella* spp. (≤ 8 % of the total). *Micrococcus cryophilus* is a Gram-negative coccus and resembles *Acinetobacter* spp. in some respects (Russell, 1974) but not in others (Juni, 1972; Jantzen *et al.*, 1975). It may be significant, therefore, that *M. cryophilus* contains a high proportion of di-unsaturated wax esters (41 to 84 % of the total) throughout its growth temperature range.

Wax esters represent a significant proportion of the lipid in cellular membranes of *M. cryophilus* (Russell, 1974). Thus, the growth temperature-dependent changes in chain length and unsaturation of wax esters may be important factors in the control of membrane fluidity in this bacterium. As the growth temperature was decreased the wax esters became more unsaturated and their average chain length decreased. Both effects would serve to maintain membrane fluidity at the lower growth temperature (Lee, 1975; Chapman & Wallach, 1968). However, the interpretation may not be so simple. Firstly, at any one growth temperature the chain length of the mono-unsaturated wax esters was relatively shorter than that of the di-unsaturated wax esters, which would tend to reduce the difference in their melting points and thus minimize differences in their effect on membrane fluidity. Secondly, the melting point of mono-unsaturated wax esters depends on whether the double bond is in the fatty alcohol or fatty acid moiety, as well as the chain length of the moiety containing the double bond; the melting point of a *cis*-mono-unsaturated wax ester is lower when the double bond is in the fatty acid than when it is in the fatty alcohol. For example, if isomers of $C_{36:1}$ wax esters are considered, the melting point of stearyl palmitoleate (18:0–16:1) is 1.5 °C lower than that of oleyl palmitate (18:1–16:0). An even greater effect on the melting point is observed when the relative chain length of the fatty alcohol and fatty acid are changed. Thus, the melting point of palmityl oleate (16:0–18:1) is 10.5 °C lower

than that of oleyl palmitate (18:1–16:0). The isomeric composition of each wax ester is therefore of crucial importance in determining its contribution to membrane fluidity, particularly since the melting points span the growth temperature range.

When the fatty alcohol and fatty acid compositions were used to generate a theoretical random wax ester mixture the composition obtained corresponded closely to that observed for intact wax esters, suggesting that specific fatty alcohol–fatty acid combinations do not occur. Similar random combinations of fatty alcohols and fatty acids have been suggested for the wax esters of the surface lipids of *Pisum* leaves (Kolattukudy, 1970) and in some marine organisms (Nevenzel, 1970) but not others (Iyengar & Schlenk, 1967). However, it is possible that, in *M. cryophilus*, in addition to the overall changes in wax ester chain length and degree of unsaturation, membrane fluidity might be regulated by alterations in the combinations of fatty alcohols and fatty acids in mono-unsaturated wax esters, which represent 15 to 50 % of the total wax esters. Such changes could be investigated by determining the fatty alcohol and fatty acid composition of individual wax esters. This is currently being studied using capillary g.l.c./mass spectrometry. It is not known what effect the presence of significant amounts of *cis* $\Delta 11$ isomers, in the fatty alcohols in particular, will have on wax ester fluidity, since there are no physicochemical data available for comparison.

The presence of *cis* $\Delta 11$ isomers poses some interesting questions about the biosynthesis of wax esters. It is assumed that, since *M. cryophilus* synthesizes unsaturated fatty acids using an aerobic $\Delta 9$ desaturase (Russell, 1978*b*), the $\Delta 11$ isomers must be synthesized by elongation of $\Delta 9$ isomers. This elongation system obviously must use an unsaturated substrate, presumably acyl-CoA, in contrast to the elongation system believed to be responsible for the modification of phospholipid acyl chain length (Russell & Sandercock, 1980), which uses a saturated substrate, because phospholipid acyl chains are almost exclusively *cis* $\Delta 9$ isomers (Russell, 1978*b*). The wax ester fatty alcohols contain a higher proportion of $\Delta 11$ isomers relative to $\Delta 9$ isomers than do the wax ester fatty acids. The basis of this difference may reside in the specificity of either the acyl-CoA reductase or the wax ester synthetase, which would synthesize and esterify, respectively, *cis* $\Delta 11$ fatty alcohols in preference to *cis* $\Delta 9$ fatty alcohols. It will be of interest, therefore, to isolate the enzymes of wax ester synthesis and to study properties such as the effect of temperature on their substrate specificity.

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