Structure, Distribution and Function of Wax Esters in *Acinetobacter calcoaceticus*

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The wax esters of *Acinetobacter calcoaceticus* strains NCIB 8250 and NCIB 10487 harvested at stationary phase from N-limited batch cultures were extracted and shown to consist of C_{14} to C_{18} saturated and mono-unsaturated alkan-l-ols randomly esterified with C_{14} to C_{18} saturated and mono-unsaturated fatty acids. The mono-unsaturated components contained a *cis* Δ9 double bond. Wax ester content of strain NCIB 8250 increased under conditions of low growth rate in N-limited continuous culture with carbon and energy source in excess. The high content of wax ester in N-limited cultures of strain NCIB 8250 was lowered by incubation in the absence of a carbon and energy source and the wax ester was converted to water-soluble materials and CO_2. It is proposed that in *A. calcoaceticus* NCIB 8250 the endogenous wax ester present in N-limited cells can serve as an energy reserve. All 19 strains of *A. calcoaceticus* tested contained some wax ester and as 16 of these strains had increased wax ester contents when harvested from stationary phase N-limited batch cultures, it appears that wax esters are widespread, but not universal, energy storage components in the genus *Acinetobacter*.

INTRODUCTION

Simple waxes, esters of unsubstituted fatty acids and unsubstituted alkan-l-ols, have not been regarded as common or major bacterial constituents (Albro, 1976). Except for studies of wax esters produced during alkane oxidation (e.g. Finnerty & Kallio, 1964; Klug & Markovetz, 1971), there have been few quantitative investigations of this type of bacterial wax. Much of the available information on *Escherichia coli* (Naccarato *et al.*, 1972), *Serratia marcescens* and *Bacillus cereus* (Kates *et al.*, 1964), *Nocardia* sp. (Hobbs *et al.*, 1971) and *Thiobacillus thioparus* (Christopher *et al.*, 1980) has been restricted to providing evidence of the occurrence of wax esters in these organisms and showing that they are trace constituents (0·02 to 0·2% of the bacterial lipid). It has been shown that wax esters occur in significant amounts in '*Micrococcus cryophilus*' (Russell, 1974), a Gram-negative coccus of uncertain taxonomic status.

In addition to these scattered observations on a wide variety of unrelated species, there have been extensive taxonomic studies of bacterial wax esters in the genera *Acinetobacter*, *Moraxella* (*Branhamella*) and *Neisseria* (Gallagher, 1971; Bryn *et al.*, 1977; Fixter & McCormack, 1976). It thus appears that only Gram-negative cocci contain substantial amounts of wax esters. The work presented in the present paper extends these observations and provides evidence that wax esters may function as an energy reserve in the genus *Acinetobacter*. Some of these results have been published in a preliminary form (Fixter & Fewson, 1974; Fixter & McCormack, 1976).

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Abbreviation: SCOT column, support coated open tubular column.

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METHODS

Strains. Most strains of *Acinetobacter* used in this study (see Table 6) have been classified in the scheme published by Baumann *et al.* (1968). EBF strains 65/65 and 65/174 were a gift of Dr A. Vivian (Bristol Polytechnic, UK) and the other strains were obtained from the appropriate culture collections. All strains were shown to be oxidase negative.

Growth and maintenance. Strains were maintained in nutrient broth cultures. Bacteria were grown at 30 °C in minimal media based on those described by Fewson (1967). In general, for large scale batch cultures inocula were prepared by transfer of 0·1 ml overnight nutrient broth culture into 100 ml succinate/salts medium (2 g KH_2PO_4 l^{-1}, 1·0 g $(NH_4)_2SO_4$ l^{-1}, 1·18 g succinic acid l^{-1}, pH 7·0) to which had been added 2·0 ml 2% (w/v) sterile $MgSO_4.7H_2O$. After overnight growth on a rotary shaker, the inoculum was transferred aseptically to 4 l of either N-limited or C-limited medium in a 10 l flat-bottomed flask and 80 ml 2% (w/v) $MgSO_4.7H_2O$ was then added. The N-limited medium contained 2·0 g KH_2PO_4 l^{-1}, 0·1 g $(NH_4)_2SO_4$ l^{-1} and 1·18 g succinic acid l^{-1}, pH 7·0. The C-limited medium contained 2 g KH_2PO_4 l^{-1}, 1·0 g $(NH_4)_2SO_4$ l^{-1} and 0·118 g succinic acid l^{-1}, pH 7·0. The culture was grown for 19 h while being stirred vigorously and aerated (Harvey *et al.*, 1968). Then 1 l sterile solution containing 4·72 g succinic acid and 8 g KH_2PO_4, pH 7·0, was added to the N-limited medium, and 1 l sterile solution containing 4 g $(NH_4)_2SO_4$ and 8 g KH_2PO_4, pH 7·0, was added to the C-limited culture. These additions were made to ensure the appropriate limitations. Bacteria were harvested, 2 h after this addition, by centrifugation at 4 °C, and the bacterial pellet was resuspended in 10 mM-potassium phosphate buffer, pH 7·0, at 4 °C.

The washed bacterial pellets were either used immediately or stored at −15 °C. Strains ATCC 17976, NCTC 10307, NCTC 10308, NCTC 10309 and NCIB 9689 did not grow well on succinate and these strains were grown in acetate/salts medium which contained 2·72 g or 0·272 g of sodium acetate l^{-1} as appropriate. That specific limitation by N or C source was obtained with media of the compositions used was checked initially in small scale cultures. After cessation of growth in limited media, growth could be re-started only by addition of the limited nutrient. Further checks were made that limitation was achieved in the large scale batch cultures (4 l). In the case of N-limited media, it was shown that NH_4^+, the N source, was not detectable in the medium and that aeration was sufficient to prevent O_2 limitation. Addition of media 2 h before harvesting was an added precaution to ensure that other nutrients were in excess. Measurements of the OD_{500} of such cultures showed that the cultures were in stationary phase and that no growth occurred after the additions of media before harvesting.

Continuous culture of strain NCIB 8250 was done essentially as described by Beggs & Fewson (1977) except that the N-limited medium contained 2·0 g $(NH_4)_2SO_4$ and 23·62 g succinic acid l^{-1}, and the C-limited medium contained 20 g $(NH_4)_2SO_4$ and 11·81 g succinic acid l^{-1}.

Extraction and purification of wax esters. Total lipids were extracted from bacteria by the procedure of Vorbeck & Marinetti (1965). After removal of non-lipid contaminants by the method of Folch *et al.* (1957), the organic solvent was removed *in vacuo* under N_2 at 45 °C. The lipid residue was dissolved in a small volume of chloroform and applied to a 0·5 mm thick Silica Gel G60 TLC plate (Merck) which was then developed with benzene (early experiments) or toluene (later experiments). After development and allowing the solvent to evaporate, areas containing lipid were located using 2′,7′-dichlorofluorescein (Skipski & Barclay, 1969). The wax-ester-containing areas were identified by comparison with the R_F value of hexadecanoyl hexadecanoate. Areas of silica gel containing the wax esters were transferred to small sintered glass filter funnels (porosity grade 4) and the wax esters were eluted three times with 2 ml chloroform. Experiments using radioactive wax esters showed that recoveries of wax esters were greater than 95% using this method. After evaporation of the chloroform the purified wax esters were dissolved in a small volume of toluene for GLC analysis.

Characterization and quantification of wax esters. GLC analysis of the wax ester fraction in terms of saturated and unsaturated components was done using a 15·2 m support coated open tubular (SCOT) column with a stationary phase of Dexsil 300 fitted in a Perkin-Elmer F17 gas chromatograph (Chromatography Services Ltd). A temperature gradient of 1 °C min^{-1} from 280 to 340 °C was used. For GLC/MS the same column and temperature programme were used in a Model 16F VG Micromass gas chromatograph–mass spectrometer (VG Micromass Ltd) which was operated by Dr R. A. Anderson, Department of Forensic Science, Glasgow University, UK.

For routine quantitative analysis, a known amount of heptadecanoyl hexadecanoate dissolved in toluene was added as an internal standard at the beginning of wax ester extraction. GLC was done using a Perkin-Elmer F11 gas chromatograph fitted with a 60 cm × 1·8 mm column packed with 3% (w/w) Alltech PS-300 (Alltech) on 100–120 mesh Gas Chrom Q (Chromatography Services Ltd) and operated isothermally at 230 °C. The amount of wax ester in the sample was determined by measuring the area of the individual wax ester component peak relative to the area of the peak of the internal standard. This GLC method was only capable of resolving wax esters containing differing numbers of C atoms.

Characterization and analysis of wax fatty acids and alkan-1-ols. Wax esters were converted to fatty acid methyl esters (FAME) and alkan-l-ols by transesterification with 2% (v/v) H_2SO_4 in methanol at 70 °C for 3 h (Christie, 1975). The FAME and alkan-l-ols were separated by TLC on Silica Gel G60 using light petroleum (b.p. 40 to 60 °C)/diethyl ether/formic acid (150:50:1, by vol.) and were located and isolated as described for wax esters.

Alkan-l-ols were converted to their acetates (Kates, 1975). GLC analysis of both FAME and alkan-l-ol acetates was done isothermally at 180 °C using a Perkin-Elmer F11 gas chromatograph fitted with a 2 m × 1·8 mm (i.d.) stainless steel column packed with 10% (w/w) Silar 10C (Chromatography Services Ltd) on 100–120 mesh Gas Chrom Q.

For determination of double bond positions in fatty acids, wax ester FAME were prepared as described above and saponified by refluxing with 1 M-KOH in 95% (v/v) ethanol for 1 h. After acidification of the saponification mixture, the fatty acids were extracted with diethyl ether. Oxidation of unsaturated fatty acids and alcohols by permanganate–periodate was carried out as described by Christie (1975) and the dicarboxylic acid fraction was analysed by GLC after conversion to methyl esters as described above. Double bond positions were also determined by conversion of the unsaturated compounds to their trimethylsilyl ethers followed by GLC/MS of these derivatives (Capella & Zorzut, 1968).

Preparation of bacteria containing ^{14}C-labelled wax esters. Cultures of strain NCIB 8250 were grown in succinate/salts medium. The bacteria were resuspended in 10 mM-potassium phosphate, pH 7·0, at a bacterial protein concentration of 200 μg ml^{-1}. A bacterial suspension (2 ml) was incubated with 2 ml [1-^{14}C]palmitic acid 1 μCi; 13·4 mCi $mmol^{-1}$ (37 kBq; 496 MBq $mmol^{-1}$)], 3 ml 100 mM-sodium succinate, pH 7·0, and 3 ml 10 mM-potassium phosphate buffer, pH 7·0, in a 25 ml conical flask at 30 °C for 30 min in a shaking water bath. Labelled bacteria were recovered by centrifugation at 4 °C and were washed three times by resuspension in 10 ml 10 mM-potassium phosphate, pH 7·0, and by centrifugation at 4 °C. The washed and labelled bacteria were resuspended at a bacterial protein concentration of 200 μg ml^{-1} in either 10 mM-potassium phosphate, pH 7·0, or this buffer containing 10 mM-$(NH_4)_2SO_4$.

Measurement of catabolites produced by ^{14}C-labelled bacteria. A labelled bacterial suspension (2 ml) was added to a 10 ml conical flask with a centre well containing 0·2 ml 14% (w/v) NaOH; the flask was sealed with a rubber seal and then incubated at 30 °C for 2 h in a shaking water bath. Metabolism was stopped by the injection of 0·2 ml 2·5 M-H_2SO_4 through the rubber seal and the incubation was continued for a further 30 min. The $^{14}CO_2$ produced was determined by counting a known volume of the 14% (w/v) NaOH in a scintillation fluid containing 3·85 g PPO l^{-1} in 23% (v/v) ethanol in toluene. Samples (1 ml) of the incubation medium were extracted by the method of Bligh & Dyer (1959). Radioactivity in water-soluble material was determined by counting a sample of the upper aqueous layer produced during the extraction. Lipids from the lower chloroform layer were separated into waxes, phospholipids, etc, by TLC on Silica Gel G60 with light petroleum (b.p. 40 to 60 °C)/diethyl ether/formic acid (150:50:1, by vol.). Areas of silica gel containing lipids were transferred to vials. The water-soluble material and lipids were counted in a scintillation fluid containing 4 g PPO l^{-1} and 0·1 g POPOP l^{-1} in 33% (v/v) Triton X-100 in toluene. In experiments using labelled compounds, the channels ratio method was used to determine the efficiency of counting. [U-^{14}C]Toluene of known specific activity was added to 10 ml of each of the scintillation fluids and varying degrees of quenching were obtained by adding choroform. Channels ratio and efficiency of counting were then determined.

Total lipid and lipid phosphorus determinations. Total lipid was determined gravimetrically and lipid phosphorus was determined by the method of Bartlett (1959) on samples of the total lipid extract produced as described for the extraction and purification of wax esters.

Bacterial dry weight. This was determined by drying at 105 °C to constant weight.

Chemicals. Wax ester standards for GLC that were not commercially available were prepared by the method of Baer & Fisher (1945) for saturated wax esters and, for unsaturated wax esters, by a modification of the method of Selinger & Lapidot (1966) for preparing fatty acid anhydrides. In the modification, 10 mmol dicyclohexcarbodiimide in 50 ml dry CCl_4 was added to a solution of 10 mmol alkan-l-ol and 10 mmol fatty acid in 150 ml dry CCl_4. After standing the solution at room temperature for 18 h, the precipitate of dicyclohexylurea was removed by filtration and the solvent was removed *in vacuo* under N_2 at 45 °C. Wax esters were purified from the lipid residue by column chromatography using Florisil as described by Caroll (1976).

Other chemicals used were Analar or were the best commercially available grades.

RESULTS

Structure of the wax esters of A. calcoaceticus NCIB 8250 and NCIB 10487

The composition of wax esters isolated from N-limited stationary batch cultures of *A. calcoaceticus* NCIB 8250 and NCIB 10487 are shown in Table 1. The wax ester compositions of the two strains were very similar, with saturated wax esters of 32 and 34 C atoms predominating. Di-unsaturated wax esters were a small proportion of the total and wax esters containing an odd number of C-atoms were present in just detectable amounts (about 0·1% by wt of the total). The effective absence of a 33 C-atom component in these analyses and in similar analyses in all other strains justified the use of a 33:0 wax as internal standard for quantitative analysis. The identity

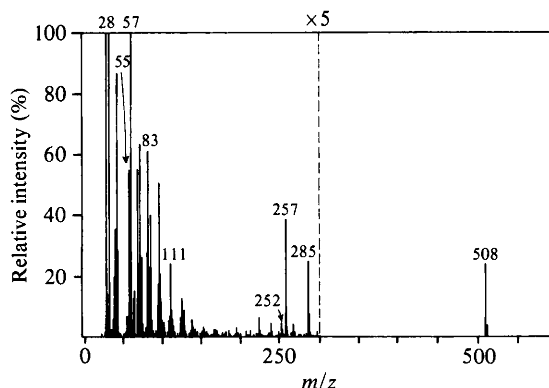

Fig. 1. Mass spectrum of 34 C-atom wax esters from N-limited stationary phase batch culture of *A. calcoaceticus* NCIB 8250.

Table 1. *Composition of wax esters from N-limited stationary phase batch cultures of A. calcoaceticus NCIB 8250 and NCIB 10487*

Wax ester analysis was done with a 15·2 m SCOT column with Dexsil 300 as the stationary phase. Wax ester components were identified by comparison of their retention times with those of authentic standards and from their mass spectra. The results are means of duplicate experiments which agreed within 10% for minor components and 5% for major components. Tc, trace (< 1% of the total).

	Composition (percentage by weight) of total wax esters	
Wax ester	*A. calcoaceticus* NCIB 8250	*A. calcoaceticus* NCIB 10487
30:0	2·9	2·2
30:1	0·4	0·6
31:0	Tc	Tc
32:0	44·9	33·3
32:1	4·8	9·8
32:2	0·4	0·8
33:0	Tc	Tc
34:0	26·9	23·1
34:1	8·5	13·6
34:2	1·9	1·9
35:0	Tc	Tc
36:0	4·0	5·7
36:1	3·1	6·2
36:2	2·1	2·8

of each component was established by comparison of its retention time with the retention times of standard wax esters and was confirmed by GLC/MS. The 34:0 wax ester (M_r 508) gave a parent ion peak at *m/z* 508 (Fig. 1). The GLC systems used, whether packed column or SCOT column, were not able to resolve wax ester isomers of the type C_m alkan-l-ol esterified with C_n fatty acid and C_n alkan-l-ol esterified with C_m fatty acid, but evidence of such isomers could be seen in the mass spectra. The 34:0 wax ester (Fig. 1) showed peaks at *m/z* 257 and 285 which are characteristic of palmitic (16:0) and stearic (18:0) acid residues respectively in the wax ester, whereas peaks at *m/z* 224 and 252 are from hexadecan-l-ol (16:0) and octadecan-l-ol (18:0) respectively. The intensity of such peaks allows the calculation of the composition of each peak in terms of isomers due to different alkan-l-ol–fatty acid combinations (Aasen *et al.*, 1971). The isomeric composition for particular carbon numbers is given in Table 2 for wax esters from strain NCIB 8250, together with the composition of each wax ester component predicted from random combination of the alkan-l-ols and fatty acids found in the total wax ester fraction. It

Table 2. *Isomeric composition of wax esters from N-limited stationary phase batch cultures of A. calcoaceticus NCIB 8250*

The isomeric composition of each wax ester was calculated from peaks in their mass spectra (Aasen *et al.*, 1971); the values given are for both saturated and unsaturated components containing a given number of C atoms. The isomeric composition predicted from the fatty acid and alkan-l-ol composition (Table 3) by random combination is also given. ND, Not detected.

No. of C atoms			Isomeric composition (moles per 100 mol wax ester)	
Wax ester	Alkan-1-ol	Fatty acid	Determined from GLC/MS	Predicted from composition
30	14	16	2·7	4·0
	16	14	97·3	96·0
32	14	18	2·3	1·6
	16	16	96·5	98·3
	18	14	1·2	0·1
34	16	18	61·0	50·4
	18	16	39·0	49·6
36	18	18	95·9	100
	20	16	4·1	ND

Table 3. *Fatty acid and alkan-1-ol composition of the wax esters and phospholipid of A. calcoaceticus NCIB 8250*

Composition was determined in N-limited stationary phase batch cultures. The results are means of duplicate experiments in which the values for major components agreed within 5% and for minor components within 10–18%. Tc, trace.

	Composition (mol%)		
	Wax ester		
Component	Fatty acid	Alkan-l-ol	Phospholipid fatty acid
12:0	Tc	Tc	2·8
14:0	0·1	2·3	2·5
16:0	55·7	58·2	35·3
16:1	12·3	7·6	17·2
18:0	18·4	23·3	1·1
18:1	13·5	8·6	41·1

can be seen that the isomeric composition determined by GLC/MS approximates closely to that predicted by random combination of the alkan-l-ols and acids found in the wax esters.

The fatty acid and alkan-l-ol compositions of the wax esters and phospholipid fractions of strain NCIB 8250 are shown in Table 3. Although traces of odd numbered wax esters were detected using SCOT column GLC, numbered fatty acids and alkan-l-ols were not detected when the fatty acid and alkan-l-ol composition of wax esters and phospholipids determined using packed column GLC which proved less able to detect minor components. Similarly, a C_{20} alkanol was found in the mass spectra of the C_{36} wax ester fraction. This alkanol, which is 4% of the C_{36} alkan-l-ols, would represent only 0·16% of total alkanols, which is close to the detection limit for the GLC system used. The alkan-l-ols contained a greater proportion of saturated components than did the wax ester fatty acids. The chain length and degree of unsaturation of wax ester fatty acids and alkan-l-ols showed marked differences from those of the fatty acids of the phospholipid fraction. Whereas octadecenoic acid (18:1) was the most abundant fatty acid in the phospholipid fraction, C_{18} mono-unsaturated components constituted only about 10% of the fatty acid and 10% of the alkan-l-ol species in the wax esters. Octadecanoic acid (18:0),

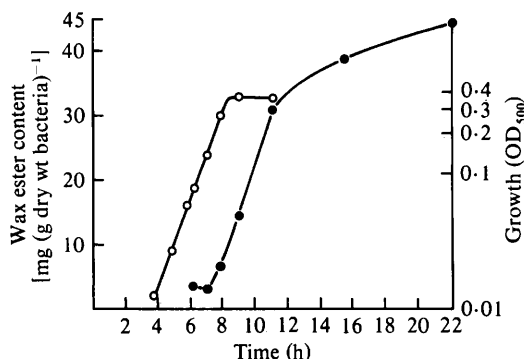

Fig. 2. Accumulation of wax esters in *A. calcoaceticus* NCIB 8250 at the transition between exponential growth and stationary phase in N-limited batch culture. ●, Wax content; ○, growth .

Table 4. *Total lipid, wax ester and phospholipid content of A. calcoaceticus NCIB 8250 in batch cultures*

Total lipid was determined gravimetrically, phospholipid phosphorus by the method of Bartlett (1959) and wax ester by GLC of lipid extracts. The values in parentheses are the number of cultures which were analysed. ND, Not determined.

	Composition [mg (g dry wt bacteria)$^{-1}$]		
Culture condition	Total lipid	Wax ester	Phospholipid-P
Exponential phase	ND	5·82 (2)	ND
C-limited stationary phase	48·7 ± 4·1 (3)	1·23 ± 0·36 (3)	2·26 ± 0·17 (3)
N-limited stationary phase	112 ± 15·0 (3)	56·3 ± 7·3 (3)*	2·29 ± 0·14 (3)

* $P < 0{\cdot}01$ for difference between N-limited and C-limited stationary phase bacteria.

which constituted 1% of the phospholipid fatty acids, occurred at a much higher concentration in the wax ester fraction.

Oxidation of the fatty acid and alkan-l-ol fractions by permanganate–periodate yielded a dicarboxylic acid fraction that contained 95% azelaic acid [$HOOC(CH_2)_7COOH$]. This showed that C_{16} and C_{18} mono-unsaturated fatty acids and alkan-l-ols contained a double bond at position 9. The octadecenoic acid was oleic acid (18:1Δ9) and the hexadecenoic acid was palmitoleic acid (16:1Δ9). GLC/MS of trimethylsilyl ethers prepared from the mono-unsaturated fatty acids and fatty acids derived from the mono-unsaturated alkan-l-ols gave for the C_{16} species intense peaks at m/z 187 and 259 respectively, and for the C_{18} species there were intense peaks at m/z 215 and 259 respectively. This is consistent with a double bond at position 9 in both C_{16} and C_{18} fatty acids and alkan-l-ols (Capella & Zorzut, 1968). As infra-red spectroscopy of the wax esters did not detect the intense band at 10·3 μm characteristic of *trans* unsaturated compounds (Christie, 1975), it is assumed that the double bonds were in the *cis* configuration.

Wax ester content of strain NCIB 8250 in N-limited cultures

The wax ester contents of *A. calcoaceticus* NCIB 8250 grown in batch cultures with succinate as carbon source are shown in Table 4. Wax ester content was increased approximately fiftyfold in stationary phase N-limited cultures compared with stationary phase succinate-limited cultures. The increased content of wax esters in N-limited batch cultures occurred at the transition between exponential growth and stationary phase (Fig. 2) when the bacterial wax ester content increased rapidly from about 2 to about 32 mg (g dry wt)$^{-1}$, and then increased much more slowly to about 45 mg (g dry wt)$^{-1}$. The wax ester content of this strain grown in

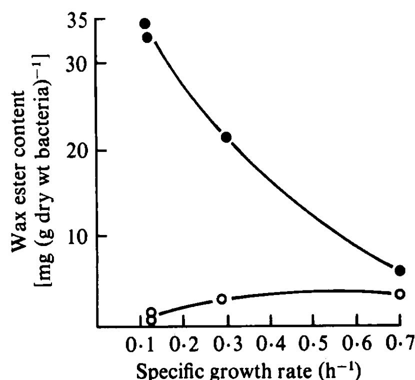

Fig. 3

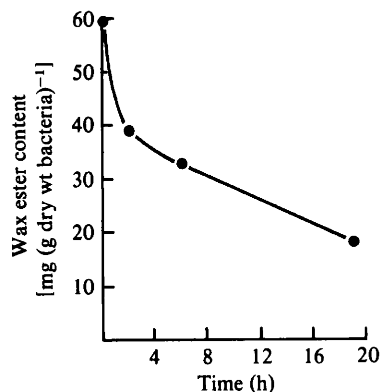

Fig. 4

Fig. 3. Wax ester content of *A. calcoaceticus* NCIB 8250 grown in continuous culture. The wax ester content was determined for cultures grown at different growth rates under N-limiting conditions (●) or C-limiting conditions (○).

Fig. 4. Wax ester content of *A. calcoaceticus* NCIB 8250 during C-starvation. N-limited stationary phase bacteria were harvested and resuspended in 10 mM-potassium phosphate, pH 7·0. The suspension was stirred and aerated at 30 °C. Samples were removed and the bacterial wax ester content determined by GLC.

Table 5. *Metabolism of radioactively labelled lipids in A. calcoaceticus NCIB 8250 during C-starvation*

Bacteria grown to N-limited stationary phase were labelled with [1-^{14}C]palmitic acid (4·12 kBq were present initially in each experimental incubation), and were suspended in 10 mM-potassium phosphate, pH 7·0, or 10 mM-potassium phosphate with $(NH_4)_2SO_4$ as described in Methods, and incubated at 30 °C for 2 h. The results are the means of two separate experiments. Total recovery of radioactivity was 85–95% of that present initially.

	Distribution (%) of total radioactivity		
	Initial	Starved	Starved + NH_4^+
Wax esters	61	1	1
Phospholipid	34	5	5
Water-soluble material	5	58	50
CO_2	0	36	44

continuous culture with either N- or C-limited media varied with the growth rate (Fig. 3). There was a significant accumulation of wax esters only at low growth rates in N-limiting conditions, e.g. 32 mg wax esters (g dry wt)$^{-1}$ at a growth rate (μ) of 0·1 h^{-1}. This is in good agreement with the results from the batch culture experiment (Fig. 3) where the content of wax esters in stationary phase (μ 0 h^{-1} nominally) was about 45 mg (g dry wt)$^{-1}$. At high growth rates (μ approximately 0·7 h^{-1}), both C- and N-limited cultures contained approximately the same amount of wax ester, which was about the same as that found in batch cultures growing exponentially with specific growth rates of approximately 0·7 h^{-1}.

Metabolism of wax esters during starvation

When bacteria grown in N-limited conditions were starved of carbon and energy, their wax ester content was lowered (Fig. 4). Bacteria labelled with [1-^{14}C]palmitic acid contained both radioactive phospholipids and wax esters, and these components were degraded during carbon and energy source starvation (Table 5). There was extensive conversion of labelled wax esters into water-soluble metabolites and CO_2 and the extent of conversion was the same in the presence and absence of exogenous NH_4^+.

Table 6. *Wax ester content of strains of A. calcoaceticus*

Wax ester content was determined for each strain in both C- and N-limited batch cultures. The results are means of duplicate experiments. The groups and subgroups of each strain in the classification of Baumann *et al.* (1968) are given in parentheses. The reproducibility of these results varied with total wax ester content, the duplicate determinations agreeing within 13–15% at wax ester contents between 0·9 and 5 mg (g dry wt bacteria)$^{-1}$ and 2–6% at wax ester contents between 35 and 140 mg (g dry wt bacteria)$^{-1}$.

Strain	Wax ester content [mg (g dry wt bacteria)$^{-1}$]	
	C-Limited	N-Limited
Group A		
NCTC 7364 (A1)	2·8	55·7
NCIB 9205 (A1)	5·3	36·8
ATCC 14290 (A1)	3·3	65·9
NCIB 8250 (A2)	1·2	56·3
ATCC 17976 (A3)	10·7	125·7
ATCC 23055* (A)	3·5	47·8
Group B		
NCTC 10307 (B1)	1·8	24·2
NCTC 10308 (B1)	2·8	1·6
NCIB 8154 (B2)	4·7	63·3
NCIB 9689 (B2)	2·5	36·4
NCTC 10304 (B3)	3·5	65·2
NCTC 10309 (B3)	0·7	12·5
NCTC 10305 (B3)	16·7	82·1
NCTC 10306 (B4)	2·3	35·2
NCIB 9115 (B4)	0·9	0·6
Unassigned		
NCIB 10487	56·5	141·4
NCIB 10553	6·8	61·5
EBF 65/65	4·8	113·6
EBF 65/174	2·9	56·7

* Neotype strain.

Wax ester content of other Acinetobacter strains grown in N-limited or C-limited conditions

The wax ester content of a series of strains grown to stationary phase in N- or C-limited conditions are shown in Table 6. The wax ester content of C-limited bacteria was generally 1 to 5 mg (g dry wt)$^{-1}$. As with strain NCIB 8250 this in general represented only a small proportion of the total lipid [40 to 120 mg (g dry wt)$^{-1}$ for these strains]. The wax ester content of the N-limited stationary phase cultures of most strains was increased five- to twentyfold compared with C-limited cultures. Strains ATCC 17976, NCTC 10305 and NCIB 10487 had the highest wax ester contents under N-limited and C-limited conditions. Wax esters were found in all the strains tested and these strains included representatives of all the subgroups of the A and B groups defined by Baumann *et al.* (1968). The composition of wax esters from representative strains, in terms of number of C-atoms only, are shown in Table 7. Their overall composition was similar whether the strains were grown in C-limited or N-limited conditions; C_{30}, C_{32}, C_{34} and C_{36} wax esters were always the major components.

DISCUSSION

The wax esters of *A. calcoaceticus* NCIB 8250 and NCIB 10487 are similar to those in *Neisseriaceae* reported by Bryn *et al.* (1977), but contain a much lower content of unsaturated wax esters compared to the *Acinetobacter* strains in the study of Bryn *et al.* (1977), and differ significantly from those reported for several *Acinetobacter* strains by Gallagher (1971) who found significant amounts of short chain wax esters. These differences may simply reflect

Table 7. *Wax ester composition of selected strains of A. calcoaceticus*

Composition was determined by GLC using a 0·6 m column of 3% PS-300 on Gas Chrom Q. This method only resolves components of different numbers of C atoms. The groups in the classification of Baumann *et al.* (1968) are given in parentheses. The results are means of duplicate experiments. The values of the duplicates were within 4–8% for major components and for minor components 5–15%. The other wax esters found in cultures were of two groups, C_{22}, C_{24}, C_{26} and C_{28} which were the largest components and C_{29}, C_{31}, C_{33} and C_{35}.

			Composition (percentage by weight) of total wax esters				
Strain	Limitation	No. of C atoms in wax . . .	30	32	34	36	Other
NCTC 7364 (A1)	C		7·1	50·0	32·1	3·6	7·1
	N		2·9	58·3	32·1	6·3	0·4
ATCC 17976 (A3)	C		3·3	31·8	44·9	19·6	2·8
	N		1·4	21·4	42·8	34·1	0·3
NCIB 8250 (A2)	C		4·0	36·0	52·0	4·0	4·0
	N		2·6	53·8	38·0	4·8	0·2
ATCC 23055* (A)	C		5·7	42·9	25·7	20·0	5·7
	N		1·5	34·1	39·1	24·5	0·8
NCTC 10307 (B1)	C		5·6	50·0	33·3	5·6	5·6
	N		0·8	17·8	47·5	33·1	0·4
NCIB 8154 (B2)	C		4·3	53·2	31·9	6·4	4·3
	N		2·4	55·3	35·7	6·3	0·2
NCTC 10309 (B3)	C		28·6	28·6	14·3	14·3	14·3
	N		8·0	52·8	35·2	2·4	1·6
NCTC 10306 (B4)	C		4·3	34·8	52·2	4·3	4·3
	N		2·0	48·0	40·1	9·7	0·3

* Neotype strain.

differences between strains and growth media used in these studies. The isomeric composition of the wax esters of strain NCIB 8250 approximates in terms of C number to that expected of a random combination of the alkan-l-ols and fatty acids which are found in the wax and this random composition was also found in the only other bacterial wax ester that has been examined in this detail (Russell & Volkman, 1980). The major mono-unsaturated alkan-l-ols and acids contain 16 or 18 C atoms which is in agreement with other reports of alkan-l-ol or fatty acid composition of *Acinetobacter* strains (Bryn *et al.*, 1977; Nishimura *et al.*, 1979). These components contain a *cis* double bond at position 9 and fatty acids containing *cis* double bonds at position 9 have been reported in a sub-group of the genus *Moraxella* (*Branhamella*) (Sugimoto *et al.*, 1983). The distribution of chain length in the wax ester alkan-l-ols and fatty acids was similar to that found in the phospholipids, but wax esters lack, or contain a very small amount of, dodecanol and dodecanoic acid and have a greatly lowered amount of mono-unsaturated C_{18} components (Table 3). These differences may arise from the specificity of the enzyme systems responsible for the reduction of fatty acids to alkan-l-ols or the specificity of the enzyme(s) catalysing the formation of the wax ester bond.

The fact that the accumulated wax esters in N-limited bacteria were degraded to water-soluble molecules and CO_2 during C-starvation, showed that they may serve as an ATP-generating substrate during starvation. This means that wax esters meet certain of the criteria for energy reserve polymers in micro-organisms (Dawes & Senior, 1973), but it remains to be seen whether a high wax ester content prevents loss of viability during starvation. Although the major energy reserves of bacteria, poly β-hydroxybutyrate, glycogen and glycogen-like materials, are polymeric and thus insoluble, wax esters are insoluble by virtue of their hydrophobic components. In C-limited bacteria, the ratio of moles of wax esters to g atoms of lipid phosphorus (which approximates to the molar ratio of wax ester to phospholipid) is about 0·04 and in N-limited bacteria this ratio is 1·6. This large change cannot be related to other

proposed functions of wax esters such as being modifiers of membrane fluidity (Russell & Volkman, 1980).

Wax esters were found in all strains of *A. calcoaceticus* examined including the neotype strain ATCC 23055. All strains except three had increased wax ester contents in N-limited cultures. This latter observation is consistent with earlier reports of substrate carbon utilization in resting cells without the accumulation of poly β-hydroxybutyrate or glycogen (Clifton, 1937; Dawes & Ribbons, 1964). Although Bryn *et al.* (1977) found wax esters in only five out of ten *Acinetobacter* strains, we have shown that the occurrence of wax esters is widespread in strains representing the full phenotypic spread of this genus.

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