Biosynthesis of Wax Esters in the Psychrophilic Bacterium *Micrococcus cryophilus*

By GEOFFREY M. LLOYD AND NICHOLAS J. RUSSELL*

Department of Biochemistry, University College, Cathays Park, P.O. Box 78,
Cardiff CF1 1XL, U.K.

(Received 11 February 1983; revised 22 March 1983)

The biosynthesis of wax esters by the psychrophilic bacterium *Micrococcus cryophilus* has been investigated using radio-gas chromatographic analysis of the constituent fatty acid and fatty alcohol moieties. Degradation studies, using α -oxidation, and the kinetics of labelling from radioactive precursors show that wax ester fatty alcohols are derived from fatty acids. Based on different labelling patterns in wax esters and phospholipids formed from acetate or a saturated fatty acid supplied exogenously, two pathways of wax ester biosynthesis are proposed. It is suggested that wax esters can be derived in the main from either a cytoplasmic or a membrane-bound pool of fatty acyl-coenzyme A thioesters and alcohols, depending on whether the fatty acid is synthesized endogenously or is supplied exogenously.

INTRODUCTION

The psychrophilic bacterium *Micrococcus cryophilus* contains relatively large amounts of wax esters, which represent 14% of the total lipid. The major wax esters are C_{36} with one (36:1) or two (36:2) double bonds, with smaller amounts of C_{32} and C_{34} components, reflecting the predominance of 18:0 and 18:1 fatty acids and fatty alcohols (Russell, 1974; Russell & Volkman, 1980). The unsaturated acids and alcohols are mainly Δ^9 isomers; smaller amounts of Δ^{11} isomers are also present, particularly in the alcohols. Changes in growth temperature lead to changes in the unsaturation, chain length and proportion of Δ^{11} isomers (Russell & Volkman, 1980). This is in direct contrast to the phospholipids, which contain exclusively Δ^9 isomers, and which respond to growth temperature changes by altering chain length, but not unsaturation (Russell, 1974). It has been argued that all of these changes are part of a mechanism whereby the bacterium regulates its membrane fluidity in response to alterations of temperature.

Although wax esters are widespread in nature, there have been relatively few studies of their biosynthesis, especially in bacteria. The fact that the combination of fatty acids and fatty alcohols in the wax esters of *M. cryophilus* is random (Russell & Volkman, 1980), suggests that the factors (e.g. temperature) that determine wax ester structure do so by regulating the nature of the acid and alcohol pools. The present paper gives the results of experiments designed to give information on the biosynthesis of the fatty acid and fatty alcohol moieties of the wax esters in *M. cryophilus*.

METHODS

Materials. Sodium [U - ^{14}C]acetate (specific radioactivity, 59 mCi mmol $^{-1}$; 2.18 GBq mmol $^{-1}$), [1 - ^{14}C]palmitic acid (specific radioactivity, 59 mCi mmol $^{-1}$; 2.18 GBq mmol $^{-1}$) and [1 - ^{14}C]stearoyl-CoA (specific radioactivity, 49 mCi mmol $^{-1}$; 1.81 GBq mmol $^{-1}$) were purchased from Amersham. Sodium acetate and stearoyl-CoA were dissolved in water and the fatty acid was dissolved in ethanol/water (1:1, v/v) and stored at $-20^\circ C$. Control experiments indicated that the amount of ethanol added (< 0.1%, by vol.) to bacterial cultures (see below) did not affect wax ester synthesis. Wax ester, fatty acid and fatty alcohol standards were obtained from Sigma.

Growth of cultures. Batch cultures of *M. cryophilus* (ATCC 15174) were grown at 20 °C (mean generation time ≈ 4 h) in a Casamino acids/salts medium to late-exponential phase of growth as described previously (Russell, 1974). These conditions maximized lipid yields whilst ensuring that growing cells were used.

Assay of wax ester synthesis. Radioactive precursors were added directly to growing cultures or to a lysate prepared by using a French pressure cell (Foot *et al.*, 1983). At timed intervals samples (1 ml) were removed and added to 3.75 ml chloroform/methanol (1:2, v/v), which stopped the incorporation of precursor, and extracted the lipids (Foot, 1980). The lipids were washed in a two-phase system (Garbus *et al.* 1963) and wax esters were isolated by preparative TLC on 0.3 mm silica gel H plates (Merck) (Russell & Volkman, 1980). Phospholipids, which remained at the origin, were also isolated (Sandercock & Russell, 1980). The wax esters were saponified and the constituent fatty acids and fatty alcohols extracted; the whole was treated to form fatty acid methyl esters, re-extracted and the esters separated from the alcohols by TLC (Russell & Volkman, 1980). The radioactivity in lipid fractions was measured by liquid scintillation spectrometry using an Intertechnique PG 4000 spectrometer with external standards ratio facility for determination of quenching.

Assay of wax ester fatty acid and fatty alcohol synthesis. Cultures were incubated with radioactive precursors, and at timed intervals aliquots were centrifuged at 12000 g ($r_{av.} = 10.7$ cm) for 10 min at 4 °C. The bacterial pellet was extracted overnight in 10 ml chloroform/methanol (1:1, v/v), and the total lipid washed using the method of Garbus *et al.* (1963). The wax esters were saponified, and fatty acids methylated and separated from fatty alcohols by TLC as described above. The isolated fatty alcohols were acetylated using acetic anhydride (Kates, 1972).

Fatty acid methyl esters and fatty alcohol acetates were analysed by radio-GLC using a Pye Unicam GCD chromatograph equipped with flame ionization detectors, and connected to a 'Panax-Reigate series' radiogas detector system. The glass columns (1.5 m $\times$ 2.0 mm i.d.) were packed with 15% (w/w) ethyleneglycol succinate silicone (EGSS-X) supported on 80–100 mesh Chromosorb W AW (Supelco, Philadelphia, U.S.A.) operated isothermally at 190 °C.

α -Oxidation studies. The α -oxidation of fatty acids or fatty alcohols, isolated from radioactive wax esters, was performed and the products analysed by radio-GLC as described previously (Sandercock & Russell, 1980). Standard errors were calculated by the method of Dean & Dixon (1951).

RESULTS

Incorporation of radioactive precursors into lipids

The radioactive precursors, sodium [U- 14 C]acetate and [1- 14 C]palmitic acid were incorporated into lipids of *M. cryophilus* extremely rapidly, i.e. within a few seconds (Fig. 1*a, b*). Radioactive labelling of phospholipid reached a plateau after 15–30 min, whereas that of wax ester peaked between 2 and 30 min (depending upon the precursor) followed by a gradual decline. The amount of incorporation of radioactive sodium acetate into wax esters was < 5% of that in the phospholipids.

The kinetics of incorporation of radioactivity from the various precursors into the fatty acids and fatty alcohols was similar to that of the intact wax esters (Fig. 2*a, b*). However, close inspection of the data shows that for each precursor, incorporation into the fatty acid reached a peak value before incorporation into the fatty alcohol (Fig. 2*a, b*).

Metabolism of wax ester fatty acids and fatty alcohols

Analysis of the constituent fatty acids and fatty alcohols of the wax esters using radio-GLC allowed changes in their chain length and unsaturation to be followed. There was a large difference in the labelling patterns obtained using these two precursors, particularly when the time course of incorporation was followed (Tables 1 and 2). Initially, acetate was incorporated into C₁₈ (mainly C_{18:0}) rather than C₁₆ fatty acids and alcohols, which were progressively desaturated to C_{18:1} (40% in fatty acid, 20% in fatty alcohol) (Table 1). In complete contrast, although much larger amounts of palmitate were incorporated, the majority remained as C_{16:0} in both fatty acid and alcohol, which were only slowly elongated and/or desaturated (Table 2). The small amount of change in fatty acid was from C_{16:0} to C_{18:1}, with little appearance of C_{16:1}, whereas the fatty alcohol showed some change from C_{16:0} to C_{16:1} together with C_{16:0} to C_{18:1}. Thus, there was a small amount of elongation and desaturation of the fatty acids and fatty alcohols synthesized from palmitate.

Fig. 1. Incorporation of sodium $[U-^{14}C]$ acetate and $[1-^{14}C]$ palmitic acid into major lipids of *M. cryophilus*. An exponentially growing culture (25 ml) was incubated at 20 °C with 125 μ Ci sodium $[U-^{14}C]$ acetate (a) or 2.5 μ Ci $[1-^{14}C]$ palmitic acid (b). At intervals samples (1 ml) were removed directly into chloroform/methanol, and the incorporation into phospholipids (●; mean values, $n = 4$) and wax esters (■; mean values, $n = 2$), determined by TLC as described in Methods. The peak values of incorporation were (a) 7.5% (822000 d.p.m.) and 0.4% (38000 d.p.m.) for phospholipid and wax ester, respectively and (b) 30.0% (66000 d.p.m.) and 8.7% (19000 d.p.m.) for phospholipid and wax ester, respectively.

Fig. 2. Incorporation of sodium $[U-^{14}C]$ acetate and $[1-^{14}C]$ palmitic acid into wax ester acids and alcohols of *M. cryophilus*. An exponentially growing culture (25 ml) was incubated at 20 °C with 125 μ Ci sodium $[U-^{14}C]$ acetate (a) or 2.5 μ Ci $[1-^{14}C]$ palmitic acid (b). At intervals samples (1 ml) were removed directly into chloroform/methanol, and the incorporation into wax acid (○; mean values, $n = 2$) and wax alcohols (□; mean values, $n = 2$) determined by TLC as described in Methods. The peak values of incorporation were (a) 0.03% (3500 d.p.m.) and 0.07% (8000 d.p.m.) for wax acid and wax alcohol, respectively and (b) 2.5% (5500 d.p.m.) and 2.0% (4500 d.p.m.) for wax acid and wax alcohol, respectively.

Table 1. *Distribution of radioactivity in wax ester acids and alcohols of M. cryophilus incubated with sodium [U-¹⁴C]acetate*

An exponentially growing culture (350 ml) was incubated at 20 °C with 175 µCi sodium [U-¹⁴C]acetate. At intervals, 50 ml or 100 ml samples were centrifuged and the wax ester acids and alcohols isolated, and analysed by radio-GLC as described in Methods. The time taken for harvesting and addition of chloroform/methanol, which stops incorporation of radioactive precursor, was 15 min, so that the time indicated is the real time of sampling.

Incubation time (min) ...	Percentage distribution of radioactivity*					
	0	15	30	60	120	240
Wax fatty acids						
Fatty acids						
16:0	9.4 ± 0.7	8.6 ± 0.5	8.0 ± 2.2	9.1 ± 2.6	8.4 ± 1.5	9.1 ± 2.8
16:1	2.3 ± 2.3	2.1 ± 2.1	3.1 ± 3.1	2.2 ± 2.2	5.2 ± 5.2	6.7 ± 6.7
18:0	83.6 ± 5.7	80.9 ± 3.7	76.8 ± 5.0	70.4 ± 13.4	56.7 ± 9.0	43.5 ± 3.0
18:1	4.8 ± 3.6	8.3 ± 1.7	12.0 ± 4.2	17.8 ± 6.1	29.6 ± 2.7	40.7 ± 3.2
Unsaturated/saturated	0.076	0.116	0.178	0.250	0.534	0.901
C ₁₈ /C ₁₆	7.55	8.34	8.30	7.41	6.36	5.33
Wax fatty alcohols						
Fatty alcohol						
16:0	6.1 ± 0.4	6.8 ± 0.2	2.6 ± 3.3	3.9 ± 4.9	3.0 ± 3.7	ND
16:1	ND	ND	ND	ND	ND	ND
18:0	94.0 ± 0.6	73.1 ± 1.2	75.0 ± 0.4	85.0 ± 18.8	68.7 ± 7.1	74.0 ± 0.3
18:1	ND	20.2 ± 1.1	22.9 ± 3.6	11.2 ± 7.0	28.4 ± 11.0	26.1 ± 0.3
Unsaturated/saturated	—	0.250	0.295	0.125	0.396	0.350
C ₁₈ /C ₁₆	15.4	13.7	37.6	24.6	32.3	0

ND, Not detected.

* Values are the mean ± S.E.M. (n = 2).

Table 2. *Distribution of radioactivity in wax ester acids and alcohols of M. cryophilus incubated with [1-¹⁴C]palmitic acid*

Cultures (300 ml) were incubated at 20 °C with 60 µCi of [1-¹⁴C]palmitic acid and treated as described in Table 1.

Incubation time (min) ...	Percentage distribution of radioactivity*					
	0	15	30	60	120	240
Wax fatty acids						
Fatty acids						
16:0	95.1 ± 1.4	94.0 ± 1.7	94.1 ± 0.6	91.6 ± 3.7	87.3 ± 4.1	84.3 ± 3.6
16:1	2.0 ± 1.2	2.4 ± 0.7	1.9 ± 0.6	2.5 ± 0.1	2.5 ± 1.7	3.3 ± 1.1
18:0	2.2 ± 1.8	2.7 ± 0.0	2.7 ± 0.6	3.2 ± 0.9	4.1 ± 0.8	3.8 ± 1.5
18:1	trace	trace	trace	2.8 ± 2.8	5.4 ± 2.9	8.2 ± 3.9
Unsaturated/saturated	0.021	0.025	0.020	0.056	0.086	0.131
C ₁₈ /C ₁₆	0.023	0.028	0.028	0.064	0.106	0.137
Wax fatty alcohols						
Fatty alcohol						
16:0	85.9 ± 3.6	75.2 ± 1.6	79.5 ± 6.8	73.5 ± 3.1	74.1 ± 5.9	63.4 ± 9.2
16:1	1.6 ± 1.2	1.5 ± 0.7	4.5 ± 2.5	7.0 ± 3.5	6.6 ± 3.5	7.8 ± 3.2
18:0	11.8 ± 4.9	20.5 ± 0.2	13.5 ± 5.0	16.7 ± 3.3	14.6 ± 4.7	13.1 ± 4.4
18:1	0.4 ± 0.4	ND	3.5 ± 1.9	2.9 ± 3.6	4.1 ± 2.6	15.0 ± 2.9
Unsaturated/saturated	0.021	0.016	0.086	0.110	0.121	0.298
C ₁₈ /C ₁₆	0.139	0.267	0.202	0.244	0.232	0.395

ND, Not detected.

* Values are the mean ± S.E.M. (n = 2).

Fig. 3. Incorporation of $[1-^{14}\text{C}]$ stearoyl-CoA into wax esters of *M. cryophilus*. Lysate (25 ml) prepared from 250 ml of a late-exponential culture as described in Methods, was incubated with $0.4 \mu\text{Ci } [1-^{14}\text{C}]$ -stearoyl-CoA. At intervals samples (1 ml) were removed directly into chloroform/methanol and the wax esters isolated by TLC as described in Methods. Values are means $\pm$ S.E.M. ($n = 2$).

α -Oxidation studies

The wax ester fatty acids and fatty alcohols isolated from bacteria incubated with sodium $[U-^{14}\text{C}]$ acetate or $[1-^{14}\text{C}]$ palmitic acid were subjected to chemical α -oxidation in order to reveal the pattern of radioactive labelling along the carbon chain. When acetate was the precursor, both acid and alcohol were labelled relatively uniformly along the length of the carbon chain. In contrast, when palmitate was the precursor the radioactivity remained at the original $1-^{14}\text{C}$ position with very little ($< 5\%$) randomization of the label.

Biosynthesis of wax esters from acyl-CoA

Incubation of a bacterial lysate with $[1-^{14}\text{C}]$ stearoyl-CoA led to a large proportion (64.1% after 10 min) being β -oxidized. There was a rapid incorporation of radioactivity into wax esters, which reached a plateau after 5–10 min (Fig. 3), when 10.9% of the added stearoyl-CoA was in wax esters. The remainder of the substrate was incorporated into phospholipids. Of the radioactivity incorporated into wax esters, approximately equal amounts were recovered from the fatty acid and fatty alcohol moieties (1090 and 973 d.p.m. per ml original lysate, respectively) after a 4 h incubation with stearoyl-CoA.

DISCUSSION

Before discussing the present data on wax ester biosynthesis in *M. cryophilus*, it is appropriate to consider the three possible metabolic routes based on studies in animal and plant tissues: (i) reversal of an esterase reaction, resulting in the esterification of a fatty acid with a fatty alcohol, (ii) acyl group transfer from a phospholipid to a fatty alcohol catalysed by an acyl transferase, and (iii) direct esterification of an activated fatty acid (e.g. acyl-CoA) with a fatty alcohol catalysed by an acyl-CoA:alcohol transacylase.

The first route, direct esterification of a fatty acid, has not been demonstrated in lipid biosynthetic pathways, including wax ester biosynthesis (e.g. see Pollard *et al.*, 1979) and so will not be considered further here, particularly in view of the following discussion.

The second route, acyl transfer from a phospholipid to a wax ester, seems unlikely in *M. cryophilus*, since the amount of radioactivity from palmitate found in wax esters decreased whilst that in phospholipids was increasing (Fig. 1*b*). Although this does not prove that there is acyl transfer in the reverse direction, i.e. from wax esters to phospholipids, it probably rules out the second route as a mode of synthesis of wax esters. Wu *et al.* (1981) also showed that jojoba seed does not synthesize wax esters at the expense of phosphatidylcholine acyl chains.

Fig. 4. Proposed routes of wax ester biosynthesis in *M. cryophilus*

It would appear, therefore, that the most probable pathway of wax ester biosynthesis in *M. cryophilus* is by an acyl-CoA:alcohol transacylase. Direct proof of this was obtained by incubating cell-free extracts with stearyl-CoA, which was incorporated readily into wax esters (Fig. 3). This is the most common mechanism for wax ester biosynthesis, being found in algae (Kolattukudy, 1970), plants (Kolattukudy, 1967), animals (Grigor & Harris, 1977), copepods (Sargent *et al.*, 1976) and another bacterium, *Mycobacterium smegmatis* (Wang *et al.*, 1972).

The kinetics of labelling from radioactive precursors (Fig. 2*a, b*) and the degradation studies using α -oxidation indicated that the alcohols were derived directly from fatty acids. The reductase that carries out this reaction in *M. cryophilus* is membrane-bound (G. M. Lloyd, unpublished results).

The two major classes of lipid in *M. cryophilus* are phospholipids and wax esters, and it is relevant to consider whether they draw acyl chains from a common or separate pools. Although acyl-CoA is a substrate for wax ester biosynthesis (see above), it does not serve as substrate for desaturation and is probably not the main donor for phospholipid biosynthesis (Foot *et al.*, 1983); the latter is probably served by acyl-ACP (Raetz, 1978), but stearyl-ACP is not converted directly to wax esters by cell-free extracts of *M. cryophilus* (Foot, 1980). Thus we can envisage an acyl-CoA 'track' leading to wax esters and an acyl-ACP 'track' leading to phospholipids, the tracks being connected by an acyl-CoA:ACP transacylase enzyme (Fig. 4).

The differences in the labelling patterns in wax esters depending on whether the precursor is acetate or palmitate can be explained on the basis of the origin of the acyl-CoA (Fig. 4). Exogenous acetate is taken up and metabolized by fatty acid synthetase to give a mixture of endogenous 16:0-ACP and 18:0-ACP. These ACP-thioesters are subject to a variety of metabolic interconversions including phospholipid biosynthesis, desaturation and formation of acyl-CoA. The latter is the substrate for wax ester synthetase, and initially after addition of radioactive acetate this produces saturated wax esters containing C_{18} (and some C_{16}) acids and alcohols. These saturated wax esters are not desaturated *per se*, and the appearance of unsaturated wax esters, containing largely $C_{18:1}$ acids and alcohols, results from the equilibration of unsaturated acyl chains from phospholipids or acyl-ACP (cf. Foot *et al.*, 1983) with the cytoplasmic acyl-CoA pool.

In contrast, exogenous fatty acids, such as palmitate, are converted directly to acyl-CoA as part of the uptake mechanism (Klein *et al.*, 1971). Since the wax ester synthetase is membrane-bound (G. M. Lloyd, unpublished results), this would provide a distinct pool of palmitoyl-CoA at the membrane for wax ester synthesis. Therefore, the addition of exogenous palmitate leads initially to saturated wax esters containing essentially C₁₆ (with very few C₁₈) acids and alcohols. Once in the wax esters, the acids and alcohols undergo little equilibration with the cytoplasmic pools of acyl-CoA and acyl-ACP. So there is much less of a time-dependent change to unsaturated wax ester (containing C_{18:1} acid and alcohol) synthesis, compared with experiments in which acetate is the precursor.

In comparison with these results, it has been suggested that in the jojoba seed there are two distinct acetyl-CoA compartments (Shine *et al.*, 1976; Ohlrogge *et al.*, 1978). Compartment I contains *de novo* fatty acid synthesis as well as palmitoyl-ACP elongation and stearoyl-ACP desaturase pathways, the entire biosynthetic sequence to oleate appearing to take place on an ACP 'track'. Unesterified fatty acid, released from this ACP 'track', is considered to be transported into compartment II where it is converted to an acyl-CoA thioester, elongated and incorporated into a wax ester.

The pathways described above for *M. cryophilus*, summarized in Fig. 4, provide an explanation of the labelling patterns observed. Although they may be an oversimplified view of the mechanisms operating *in vivo*, they provide a basis for future experiments on wax ester biosynthesis in this psychrophilic bacterium.

The authors wish to thank the SERC for financial support.

REFERENCES

- DEAN, R. B. & DIXON, W. J. (1951). Simplified statistics for small numbers of observations. *Analytical Chemistry* **23**, 636-638.
- FOOT, M. (1980). *Studies on a bacterial Δ^9 desaturase*. Ph.D. thesis, University of Wales, U.K.
- FOOT, M., JEFFCOAT, R. & RUSSELL, N. J. (1983). Some properties, including the substrate *in vivo*, of the Δ^9 -desaturase in *Micrococcus cryophilus*. *Biochemical Journal* **209**, 345-353.
- GARBUS, J., DELUCA, H. F., LOOMANS, M. E. & STRONG, F. M. (1963). The rapid incorporation of phosphate into mitochondrial lipids. *Journal of Biological Chemistry* **238**, 59-63.
- GRIGOR, M. R. & HARRIS, E. L. (1977). Wax ester synthesis in the mouse preputial gland tumour. *Biochimica et biophysica acta* **488**, 121-127.
- KATES, M. (1972). *Techniques of Lipidology. Isolation, Analysis and Identification of Lipids*, 1st edn, p. 519. Amsterdam: North-Holland Publishing Co.
- KLEIN, K., STEINBERG, R., FIETHEN, B. & OVERATH, P. (1971). Fatty acid degradation in *Escherichia coli*. An inducible system for uptake of fatty acids and further characterization of old mutants. *European Journal of Biochemistry* **19**, 442-450.
- KOLATTUKUDY, P. E. (1967). Mechanisms of synthesis of waxy esters in Broccoli (*Brassica oleracea*). *Biochemistry* **6**, 2705-2717.
- KOLATTUKUDY, P. E. (1970). Reduction of fatty acid to alcohols by cell free preparations of *Euglena gracilis*. *Biochemistry* **9**, 1095-1102.
- OHLOGGE, J. B., POLLARD, M. R. & STUMPF, P. K. (1978). Studies on biosynthesis of waxes by developing jojoba seed tissue. *Lipids* **13**, 203-210.
- POLLARD, M. R., MCKEON, T., GUPTA, L. M. & STUMPF, P. K. (1979). Studies on biosynthesis of waxes by developing jojoba seed. II. The demonstration of wax biosynthesis by cell-free homogenates. *Lipids* **14**, 651-662.
- RAETZ, C. R. H. (1978). Enzymology, genetics and regulation of membrane phospholipid synthesis in *Escherichia coli*. *Microbiological Reviews* **42**, 614-659.
- RUSSELL, N. J. (1974). The lipid composition of the psychrophilic bacterium *Micrococcus cryophilus*. *Journal of General Microbiology* **80**, 217-225.
- RUSSELL, N. J. & VOLKMAN, J. K. (1980). The effect of growth temperature on wax ester composition in the psychrophilic bacterium *Micrococcus cryophilus* ATCC 15174. *Journal of General Microbiology* **118**, 131-141.
- SANDERCOCK, S. P. & RUSSELL, N. J. (1980). The elongation of exogenous fatty acids and the control of phospholipid acyl chain length in *Micrococcus cryophilus*. *Biochemical Journal* **188**, 585-592.
- SARGENT, J. R., LEE, R. F. & NEVENZEL, J. C. (1976). Marine waxes. In *Chemistry and Biochemistry of Natural Waxes*, pp. 49-91. Edited by P. E. Kolattukudy. Amsterdam: Elsevier.
- SHINE, W. E., MANCHA, M. & STUMPF, P. K. (1976). Fat metabolism in higher plants: the function of acyl thioesterases in the metabolism of acyl coenzyme A and acyl-acyl carrier proteins. *Archives of Biochemistry and Biophysics* **172**, 110-116.
- WANG, L., TAKAYAMA, K., GOLDMAN, D. S. & SCHNOES, H. K. (1972). Synthesis of alcohol and wax ester by a cell-free system in *Mycobacterium tuberculosis*. *Biochimica et biophysica acta* **260**, 41-48.
- WU, X.-Y., MOREAU, R. A. & STUMPF, P. K. (1981). Studies of biosynthesis of waxes by developing jojoba seed. III. Biosynthesis of wax esters from acyl-CoA and long chain alcohols. *Lipids* **16**, 897-902.