Importance of stored triacylglycerols in *Streptomyces*: possible carbon source for antibiotics

Ekundayo R. Olukoshi and Neville M. Packter

Submerged cultures of four *Streptomyces* species accumulated triacylglycerol (TAG), ranging from 50 to 150 mg (l medium)⁻¹, during the post-exponential phase of growth. *S. lividans* also produced glycogen (80 mg l⁻¹). Identity of TAG species was confirmed after TLC, by mass spectroscopy and by quantitative IR spectroscopy and reaction of the hydroxamate derivatives with ferric chloride. This is the first substantive report showing the storage of TAG in bacteria. Distribution of diacylglycerol acyltransferase (TAG synthase) activity from *S. coelicolor* and *S. lividans* during incubation on different media paralleled the formation of TAG. Accumulation of TAG may be necessary to maintain cell integrity after glucose becomes exhausted from the medium, and also to provide the C₆ units needed for subsequent biosynthesis of acetate-derived antibiotics in appropriate species. Actinorhodin was formed by *S. coelicolor* when grown on YEME medium only after exhaustion of glucose; the carbon source may therefore originate from TAG. All the organisms examined in this study formed isoprenoid-derived hydrocarbons (up to 3 mg l⁻¹) which were identified as squalene plus hydrogenated derivatives.

**Keywords**: diacylglycerol acyltransferase, hydrocarbons, triacylglycerols, *Streptomyces*

**INTRODUCTION**

*Streptomyces* spp. are a group of filamentous Gram-positive bacteria found in soil. Their life cycle on solid medium involves a vegetative mycelial phase and a reproductive sporulation stage during which many strains produce antibiotics. Many of these are in current clinical or agricultural use (Berdy, 1974) and include phenolic or macrolide derivatives produced by a sequence of reactions similar to that responsible for fatty acid biosynthesis (Packter, 1980; Hopwood & Khosla, 1992). While much is known about the genetics of antibiotic formation in these organisms (Hopwood, 1967; Hopwood & Khosla, 1992), many aspects of lipid metabolism have remained unexamined (Verma & Khuller, 1983). Indeed, aspects of central metabolism in general have seldom been studied in depth (White et al., 1992) and little is known about the source of carbon for antibiotic formation.

It has frequently been stated that bacteria do not accumulate neutral lipids such as triacylglycerol (TAG) (Lennarz, 1966; Shaw, 1974; Harwood & Russell, 1984), whereas polyhydroxybutyrate (Dawes & Senior, 1973; Dawes, 1985) and glycogen (Preiss & Romeo, 1989) are typical storage polymers in these organisms. The presence of TAG, however, has been briefly reported in *Streptomyces* (Ballio et al., 1965; Verma & Khuller, 1980; Packter et al., 1985) in addition to the typical membrane phospholipids. The fatty acids present in these lipids are predominantly branched-chain of the iso- and anteiso-series plus palmitate (Ballio et al., 1965; Packter et al., 1985). No systematic studies on the formation of storage lipids during growth have been reported. In this paper, we demonstrate the formation of TAG, and also hydrocarbons, during growth of *S. lividans*, *S. coelicolor*, *S. albus* and *S. griseus* on a rich medium. We also describe the isolation from *S. coelicolor*, and distribution during growth, of a diacylglycerol acyltransferase (TAG synthase), an enzyme which catalyses the final step in the pathway of TAG synthesis. The possible relationship between exhaustion of glucose from the medium, availability of TAG and subsequent formation of actinorhodin, an acetate-derived antibiotic made by *S. coelicolor*, is also examined.

**Abbreviations**: DAG, diacylglycerol; TAG, triacylglycerol
METHODS

Materials. All chemicals, solvents and reagents were of analytical grade and were obtained from BDH, Fisons and Sigma. Reference lipids and diacylglycerol (DAG) samples were obtained from Sigma. Agar and other media components were obtained from Lab M Products. Kieselgel (60 H) adsorbent for thin-layer chromatography (TLC) was obtained from BDH and silicic acid used for column chromatography was purchased from Mallinckrodt. Radiolabelled substrates, [14C]palmitoyl-CoA and [14C]triolein, were purchased from Amersham and New England Nuclear (NEN), respectively.

Culture conditions. Streptomyces lividans J1 1126 and S. coelicolor A3(2) [and a red(−) mutant (B385) derived from this] were obtained from the John Innes Institute, Norwich, UK. S. coelicolor J802 was a gift from Dr G. Hobbs of the Manchester Biotechnology Centre, UK. S. albus and S. griseus were obtained from NCIIMB Ltd, Aberdeen, UK. Stock cultures of these organisms were grown as agar slants on the R2YE medium described by Okanishi et al. (1974) for 7 d at 30 °C and then maintained at 4 °C for up to 6 weeks. This medium was supplemented with proline and arginine (each 50 mg l⁻¹) when the red(−) mutant was used. Spores from five agar slants were gently scraped off after addition of sterile water (5 ml) to each tube and routinely used to inoculate 1 l complete medium (CM) (Hopwood, 1967) or yeast extract/malt extract (YEME) medium (Horinouchi & Beppu, 1984; Hopwood et al., 1985), after filtration through glass wool to hold back agar and hyphal debris. Liquid CM or YME medium, containing approximately 2 × 10⁶ spores ml⁻¹, was dispensed aseptically in 50 ml or 100 ml aliquots into sterile 250 ml Erlenmeyer flasks, fitted with stainless steel springs around the bottom to enhance dispersed growth; cultures were incubated on an orbital shaker (250 r.p.m.; 30 °C). Occasionally, cultures were grown initially in 21 flasks containing 500 ml medium and were incubated at 160 r.p.m. The flasks were pretreated with 5% (v/v) hexamethylen disilazone in chloroform to prevent formation of surface mycelium around the flask sides (Packter & Collins, 1974). For experiments involving analysis of hydrocarbons, mycelium from 5 × 250 ml flasks was used to inoculate 101 CM and growth continued under forced aeration at 30 °C. Silicone antifoaming agent (0.1 ml l⁻¹) was routinely added to all media. Mycelium was harvested by filtration through ‘Miracloth’ and the mycelial pad was washed twice with cold 0.1 M phosphate buffer (pH 7.2) and frozen at −70 °C overnight before being freeze-dried.

Medium transfer. Cells of S. lividans were grown to early exponential phase (12 h) and then transferred according to the following procedure. The cells were allowed to settle and the bulk of the original medium aseptically decanted. The cells were washed by resuspension in the appropriate medium at 30 °C before adding to medium of that composition. Cells were also harvested at transfer for analysis. The low-nitrogen medium contained 10% of the amount of peptone and Casamino acids present in the complete medium. Cultures were grown in duplicate.

Spore counts. Spores were counted using a Thoma chamber with dimensions 0·02 mm × 1/400 mm² (Weber Scientific).

Extraction of lipids. Total lipids were obtained from powdered freeze-dried cells by extraction with chloroform/methanol (2:1, v/v) as described by Folch et al. (1957). The lipid extract was dissolved in 0·5 ml chloroform and stored at −15 °C prior to analysis.

Methylation of lipids. The constituent fatty acids within lipids were converted into their methyl esters for mass spectral analysis (Metcalfe & Schmitz, 1961). Lipids (2–10 μmol fatty acid) in chloroform were placed in 10 × 60 mm stopped pyrex tubes and dried under nitrogen. BF₃/methanol reagent (14%, w/v) (0·2 ml) was added and heated at 90 °C for 30 min. Methyl esters were extracted into hexane after addition of water (0·5 ml), and purified by TLC.

Column chromatography. The total lipid extract was separated into neutral and polar lipids by chromatography on silicic acid (Mallinckrodt, 100 mesh; weight ratio of silicic acid to sample 30:1), with the following solvents: light petroleum (b.p. 40–60 °C, obtained after distillation), light petroleum/chloroform (1:1, v/v) then chloroform; and chloroform/methanol (1:1, v/v). This procedure eluted hydrocarbons, triacylglycerols and phospholipids respectively.

TLC. TLC was routinely carried out on Kieselgel (60 H) to confirm the identity of lipids. Chromatography was performed on 20 × 20 cm glass plates (0·25 cm thick), placed in lined tanks containing the following solvent systems: (a) light petroleum (b.p. 40–60 °C)/diethyl ether/glacial acetic acid (80:20:1 or 90:10:1, by vol.); and (b) chloroform/methanol/acetic acid/water (65:25:4:1, by vol.) for the separation of neutral and polar lipids respectively. Plates were routinely run initially in chloroform/methanol (1:1, v/v) to remove soluble impurities in the silica gel. Spots were visualized by exposure to i₂ vapour, spraying with dichlorofluorescein or with Dittmer’s reagent (in the case of phospholipids). Hydrocarbons were initially resolved as a single band using light petroleum/diethyl ether (98:2, v/v) and then further fractionated with light petroleum on either Kieselgel or AgNO₃-impregnated plates.

Assay of lipids. Initially, S. lividans and S. coelicolor [red(−)] were used for studies of lipid production during growth. The red(−) mutant does not produce undecylprodigiosin (Rudd & Hopwood, 1980), a red pigment formed earlier than actinorhodin, and therefore this would not interfere with lipid analysis at the times studied. TAG and phospholipids were assayed by the ferric hydroxamate method (Hughes & Elliott, 1969) with tripalmitin and phosphatidyethanolamine as standards. Hydrocarbons and TAG in solution in carbon tetrachloride were additionally assayed by IR spectroscopy using 1 mm cells by measuring the stretching band absorbances due to C–H (2968 cm⁻¹) and C=O (1742 cm⁻¹) respectively. The amount of lipid present was determined from calibration curves [absorbance = log 1/T% versus concentration (mg ml⁻¹)] obtained from squalene and purified TAG isolated from Streptomyces as standards; assays were linear up to 2·0 mg ml⁻¹ and 3·5 mg ml⁻¹ respectively. A double-beam Perkin Elmer IR spectrophotometer was used for these assays, which were non-destructive. Good agreement for TAG values (within 3%) was obtained between the two methods, both of which were generally used for assays at different incubation times. All assays were performed at least in duplicate and the mean value presented; they gave replicates within 3% of each other. Variation in lipid content (and in dry wt and protein) between duplicate experiments was within 6%.

Analysis of spores. Distilled water (5 ml) was added to each of 70 agar slants of S. lividans. The spores were scraped off gently with a wire loop and vortexed for 30 s. The spore suspension was filtered through glass wool and centrifuged at 4000 g for 10 min. The spore pellet was washed twice with 0·1 M phosphate buffer (pH 7·2), frozen at −70 °C and subsequently
freeze-dried. Spores were ground with acid-washed sand and subsequent lipid extraction and analysis were performed as described above.

**Extraction and assay of glycogen.** Cells were extracted and assayed for glycogen after treating freeze-dried mycelium with 30% (w/v) KOH as described by Braña et al. (1982). Precipitates obtained from this procedure were hydrolysed in sealed tubes with concentrated HCl at 100 °C for 4 h. Glucose was determined by the o-toluidine method (Dubowski, 1962) and corrected for glycogen content by multiplying by 0.9 (Sigal et al., 1964). Paper chromatographic analysis was carried out on Whatman no. 1 filter paper using ethyl acetate/pyridine/water (12:5:4, by vol.) as solvent system. Spots were visualized by passing the paper through aniline/phosphoric dip solution and placing the paper in an oven (110 °C) for 5 min, after which sugars appeared as brownish spots. The hydrolysed product gave an Rf value identical with that observed for glucose.

**Assay of polyhydroxybutyrate.** Mycelium was extracted and assayed for polyhydroxybutyrate as described by Law & Slepecky (1961) by measuring the peak absorbance at 235 nm corresponding to the presence of crotonic acid. This method permits determination of approximately 5 µg ml⁻¹ of this polymer.

**Extraction of actinorhodin.** Actinorhodin was extracted from freeze-dried mycelium by grinding with 2 M NaOH (50 ml per g mycelium) for 1 h (Wright & Hopwood, 1976). Pigment was quantitatively extracted into ethyl acetate from the acidified supernatant after centrifugation at 4000 g for 10 min. Its spectrum was recorded in methanol (1–5 mg in 5 ml) between 400 and 800 nm. A similar spectrum for the alkaline form was also obtained (blue in colour) by adding 50 µl 2 M NaOH. Amount of pigment present was calculated using an A₄₉₀ of 200 for the alkaline form.

**Estimation of protein.** Protein was isolated from the mycelium after grinding with acid-washed sand and extraction with 1 M NaOH (30 ml g⁻¹) at 40 °C for 30 min. Protein samples were precipitated from the supernatants of the centrifuged homogenates with 10% (w/v) trichloroacetic acid solution prior to estimation by the Lowry method using bovine serum albumin (BSA) as standard.

**Determination of mass spectra.** These were recorded on a VG Autospec mass spectrometer at a source temperature of 200 °C and ionization energy of 70 eV. Samples (approx. 1 mg ml⁻¹) were introduced in carbon tetrachloride. Methyl esters were readily separated from phospholipids purified by column chromatography and subsequent crystallization from ethanol, and also from phospholipids purified by column chromatography. Lipids were initially isolated from S. lividans by phospholipase C action (Hanahan & Vercamer, 1954) and purified by TLC. Assays were linear with time up to 45 min and also with protein added, when up to 200 µg protein was employed. Reaction was terminated by the addition of 1.5 ml 2-propanol. Lipids were extracted into hexane (5 ml) after addition of triolein (20 µg), evaporated to dryness under nitrogen and applied to TLC plates. These were developed in light petroleum/diethyl ether/acetic acid (80:20:1, by vol.) and the band corresponding to TAG scraped off into plastic scintillation vials. Optiphase (10 ml) was added and the radioactivity measured with a scintillation counter. It was essential to resolve the TAG by this means since the presence of an alcohol acyl transferase in the preparation gave rise to labelled alkyl esters. These were readily separated from TAG by TLC.

**Assay of TAG lipase.** Assays were carried out as described by Paznakos & Kaplan (1977) and were based on the release of oleic acid from [¹⁴C]triolein. Assays were carried out in screw-capped tubes in a total volume of 1.0 ml in a shaking water bath at 30 °C. Each system contained 50 mM phosphate buffer (pH 6.5), 1-0 mg ml⁻¹ BSA (fatty acid free), 25 mg ml⁻¹ Triton X-100 and 2 µM triolein (124 µCi µmol⁻¹) in 10 µl ethanol. Membrane protein (100–200 µg) was added to initiate the reaction, which was terminated after incubation for 1 h with 2-propanol/hexane/0.75 M H₂SO₄ (40:10:1, by vol.); 5 ml. Reaction products were extracted into hexane (5 ml), after addition of 10 µg each of triolein and oleic acid as carriers. The solvent was removed under nitrogen and the extract subjected to TLC (light petroleum/diethyl ether/acetic acid, 70:30:1, by vol.). The fatty acid band, revealed after brief exposure to I₂ vapour, was scraped off into scintillation vials and its radioactivity measured after addition of Optiphase (10 ml). The reaction rate was linear for 90 min and up to 400 µg of protein.

**RESULTS**

**Growth of organisms.**

Growth was based on the submerged cultural conditions described, using initially S. lividans and S. coelicolor [red(−)], to permit isolation of lipids without contamination by pigments. These conditions gave rise to growth that was satisfactorily reproducible and homogeneous; the cells were largely filamentous, but also comprised small colonies especially at later stages of growth. After an initial lag phase, the cultures grew exponentially as determined by measurements of dry weight, protein, and phospholipid content. Slight increases in dry weight were occasionally found after long incubation periods and this
Formation of lipids during growth

The variation in lipid content with incubation period was first investigated during growth of \textit{S. lividans} and \textit{S. coelicolor} [red(—)] in complete medium. The results of typical studies for cells incubated after inoculation with spores or after transfer of mycelium grown to early exponential phase are shown in Figs 1 and 2 respectively. Very similar results were found for each organism and were also subsequently obtained with \textit{S. coelicolor} (wild-type) or when \textit{S. albus} or \textit{S. griseus} were used. Irrespective of the type of inoculum (spores or mycelium), or the organism used, TAG was only detected in very small amounts during early exponential growth; the major lipids at this time were phospholipids. These were identified as phosphatidylethanolamine, cardiolipin and phosphatidylinositol dimannoside (Packter \textit{et al.}, 1985) and were similar to those found in other actinomycetes (Verma \& Khuller, 1983) and Gram-positive bacteria (Goldfine, 1972, 1982).

When the cells reached stationary phase (20 h after inoculation, Fig. 1, or 12 h after transfer in this medium, Fig. 2), TAG accounted for approximately 20\% of the total lipid whereas the predominant lipid type present was phospholipid. Increased lipid content after this stage (when net protein and phospholipid synthesis had ceased) was due entirely to increased TAG synthesis and accumulation in all the \textit{Streptomyces} tested. At 48 h incubation (or 24 h after transfer), TAG accounted for about 60\% of the total lipid (approx. 120 mg per l medium; 50 mg per g mycelium) (see Figs 1 and 2). The concentration of TAG increased up to approximately 60 h incubation. This pattern of TAG accumulation contrasted sharply with that observed with phospholipids, which remained steady throughout stationary phase, confirming that their function was related to cell growth and membrane turnover. Significant amounts of free fatty acids, monoacylglycerols and diacylglycerols were not

Fig. 1. Formation of lipids during growth of \textit{S. lividans}. The organism was grown in submerged culture as described in Methods in 2 l flasks, each containing 500 ml medium. Mycelium from two flasks was harvested at various times after inoculation by filtration and freeze-dried. Lipids were isolated and assayed by the hydroxamate method (TAG and phospholipids) and by IR spectroscopy. ○, Dry weight; ●, protein; □, TAG; ■, phospholipids; △, hydrocarbons. Experiments were done three times and the results presented are means of the three.

Fig. 2. Formation of lipids after transfer of growing mycelium into fresh medium. Cells of \textit{S. coelicolor} [red(—)] were grown to early exponential phase (12 h) in \textit{20} x 250 ml flasks, each containing 100 ml medium; mycelium was aseptically transferred to 5 l fresh medium held in each of two 10 l flasks at 30 °C. The cell suspension was then dispensed in 500 ml portions into 2 l flasks. Mycelium from two such flasks was harvested after further incubation for various times and assayed as described in Fig. 1. ○, Dry weight; ●, protein; □, TAG; ■, phospholipids; △, hydrocarbons. Experiments were done twice and the results presented are means of the two.

feature was more pronounced when cells were grown in YEME medium. Stationary phase commenced in complete medium approximately 20 h after inoculation or 12 h after transfer, at which time net protein and phospholipid synthesis had ceased.
Mass spectroscopic studies confirmed that the fatty acids present in TAG (and phospholipids) were predominantly C_{15}, C_{16} and C_{17} in chain length but C_{18} acids were also present. Analysis of methyl esters derived from TAG from \textit{S. lividans} showed strong peaks at \( m/z \) 74 and 87, characteristic of saturated, non-hydroxylated derivatives, corresponding to the oxygen-containing fragments \((\text{CH}_3\text{OC} = \text{CH}_2)^*\) and \((\text{CH}_3\text{OCC}_2\text{H}_4\text{CH}_3)^*\) respectively (Benyon, 1960; Ryhage & Stenhagen, 1963).

Mass peaks appeared predominantly at \( m/z \) 256, \( m/z \) 270 and \( m/z \) 284, corresponding to methyl esters of C_{15}, C_{16} and C_{17} fatty acids, approximately in the ratio 1:0:1.7:1.8; much smaller amounts of C_{18} (\( m/z \) 298) were also found (Fig. 3). A similar composition was found for phospholipids except that the major C_{15}, C_{16} and C_{17} acids were present in approximately equal amounts. Similar results overall were determined for TAG derived from \textit{S. coelicolor} and \textit{S. griseus}, however, C_{18} acids were present in much greater proportions and were predominant. These data are illustrated in Fig. 3 using material from \textit{S. lividans} and confirm earlier results from gas chromatographic studies using \textit{S. coelicolor} (Packter \textit{et al.}, 1985). This work had demonstrated the presence of \textit{iso-} and \textit{anteiso-}derivatives of C_{15}, C_{16} and C_{17} acids after characterization by GLC using suitable reference standards.

Spores from agar slopes of \textit{S. lividans} were extracted for lipids and their contents are indicated in Table 1. Analysis showed the presence of phospholipids, TAG and hydrocarbons. However, the amounts present in spores used for inoculation were considerably lower than those detected at the earliest stage of submerged growth which was examined, confirming the synthesis of these lipids during the period tested. The bulk of the hydrocarbons present in the spores could be extracted directly with cold light petroleum. Similar results were gained from lipid extracts of spores from \textit{S. coelicolor} A3(2).

Essentially similar patterns of period of exponential growth and yield of TAG were obtained from other \textit{Streptomyces} spp. examined. Results of experiments in which four different \textit{Streptomyces} species were grown in complete medium and their ability to form TAG, and to utilize glucose, were determined are presented in Table 2. At 24 h, they had all entered stationary phase as evidenced from the subsequent steady values obtained for dry weight, protein and phospholipid content. All the

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Lipid & Weight (mg) \\
\hline
Hydrocarbons & 2.2 ± 0.3* \\
Hydrocarbons & 0.2 ± 0.1 \\
TAG & 3.2 ± 0.6 \\
Phospholipids & 3.0 ± 0.5 \\
\hline
\end{tabular}
\caption{Lipid composition of spores obtained from \textit{S. lividans}}
\end{table}

Spores from \textit{S. lividans} were obtained from agar slants, frozen and freeze-dried. They were initially extracted with cold light petroleum (b.p. 40–60 °C) and subsequently with chloroform/methanol; this latter extract was resolved by chromatography on silicic acid and identity of lipids was confirmed after TLC. The experiment was performed three times, each using 70 slants, and the results presented are the means ± standard deviations.

Spores from \textit{S. coelicolor} \textit{[red(-)]} but the C_{17} product was the major acid with a trace of C_{16} present. With \textit{S. albus} and \textit{S. griseus}, however, C_{18} acids were present in much greater proportions and were predominant. These data are illustrated in Fig. 3 using material from \textit{S. lividans} and confirm earlier results from gas chromatographic studies using \textit{S. coelicolor} (Packter \textit{et al.}, 1985). This work had demonstrated the presence of \textit{iso-} and \textit{anteiso-}derivatives of C_{15}, C_{16} and C_{17} acids after characterization by GLC using suitable reference standards.

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Table 2. Glucose utilization and TAG synthesis by Streptomyces species

Organisms were grown in submerged culture and harvested by filtration at the times indicated. Lipids were isolated from mycelium (produced by two flasks each containing 100 ml complete medium) after extraction with chloroform/methanol, and assayed by IR spectroscopy and the hydroxamate method. Residual glucose in the medium was determined by the o-toluidine method (ND, < 0.5 g). Experiments were done twice and the results presented are means of the two, expressed per 1 medium. Variations in lipid content and other parameters agreed to within 6%.

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<th>Organism</th>
<th>Incubation period (h)</th>
<th>Residual glucose (g)</th>
<th>Dry weight (g)</th>
<th>Protein (mg)</th>
<th>Phospholipid (mg)</th>
<th>TAG (mg)</th>
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Table 3. Effect of nitrogen and glucose content of medium on the accumulation of TAG by S. lividans

Replicate cultures were grown in complete medium and transferred as described in Methods. Transfers were made after 12 h incubation. Cultures were harvested for analysis at the time of transfer and following additional incubation after transfer. Unchanged cultures and cultures transferred to complete medium served as normal growth controls. Results are based on three flasks per group (total of 150 ml) but expressed per 1 medium. Experiments were done three times; the results presented are representative.

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<th>Medium</th>
<th>Incubation period (h)</th>
<th>Dry weight (g)</th>
<th>Protein (mg)</th>
<th>Phospholipid (mg)</th>
<th>TAG (mg) [mg (g protein)-1]</th>
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<td>2.3</td>
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<td>250</td>
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<tr>
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</tbody>
</table>

Streptomyces species examined accumulated TAG to similar extents (up to approx. 120 mg per 1 medium). However, only S. griseus rapidly utilized glucose, with a corresponding drop in TAG at 72 h, when all the glucose in the medium had been exhausted. In the other species, glucose levels in the medium remained constant at relatively high concentrations (approx. 70% of the initial amount) for a considerable time after the cells had entered stationary
Stored triacylglycerols in *Streptomyces*

Phase. Similarly, the concentration of TAG remained steady at these times.

Cells of *S. lividans* containing minimal amounts of TAG, obtained from early exponential cultures, were transferred to modified environments in experiments aimed to determine conditions which might regulate TAG biosynthesis. Transfer to the low-nitrogen medium (see Methods) resulted in a minimal increase in dry weight but when the added nitrogen became limiting a fourfold relative increase in TAG occurred (Table 3). An even higher value of TAG accumulation was found when the cells were incubated in a nitrogen-free medium. In this situation, a fivefold increase in relative TAG content over the cells grown in complete medium (used as control) was observed. In the high (or no) nitrogen/no carbon medium, accumulation of TAG was not observed over the period tested.

### Hydrocarbon composition

Hydrocarbons were also initially detected in small amounts in *S. lividans* during the exponential phase but even the amount present at the earliest growth time tested (14 h) was significantly (approx. 10-fold) greater than that present in the spore inoculum used for those cultures (Table 1). At 48 h, hydrocarbon content had increased to 2–3 mg per l medium for this organism; similar results were noted for *S. coelicolor*, *S. albus* and *S. griseus*.

Resolution of species of hydrocarbons obtained from *S. lividans* was performed by TLC. This procedure revealed the presence of four distinct bands designated *S*₁, *S*₂, *S*₃ and *S*₄ (in increasing Rₚ values) and present in similar amounts, but with *S*₁ and *S*₃ predominant. A similar composition was found in cells at all stages of growth examined. The bands were eluted separately with diethyl ether and analysed further by mass spectroscopy; this indicated that they comprised a family of related C₁₀₀ isoprenoid compounds. Compound *S* showed spectral characteristics identical to authentic squalene, with a parent molecular ion at m/z 410, characteristic base peaks at m/z 69 [H(C₅H₇)]⁺ and 81 [H(C₅H₇)C⁺] and relatively intense fragments at m/z 136 and 137, due to the presence of [(C₅H₇)(C₅H₇)]⁺ and [H(C₅H₇)(C₅H₇)]⁺. Compounds *S*₂, *S*₃ and *S*₄ had parent molecular ions at m/z 412, m/z 414 and m/z 416 respectively, in addition to the characteristic base peaks found in squalene (Fig. 4), indicating the presence of a mixture of squalene and partially hydrogenated products (dihydro-, tetrahydro- and hexahydro-derivatives respectively) in this organism.

The major hydrocarbons found in *S. coelicolor* were the dihydro- and tetrahydro-squalenes (m/z 412 and m/z 414), whereas octahydro-squalene (m/z 418) was the predominant product in *S. griseus*. However, *S. albus* was the only species examined which contained dehydro-squalene and squalane (m/z 408 and m/z 410) in approximately equal amounts as the major products, together with smaller amounts of the di- and tetrahydro-derivatives, plus substantial amounts of the fully saturated squalane (dodecahydro-squalene) with a molecular ion at m/z 424.

Conclusive evidence for the presence of fatty-acid-derived hydrocarbons in these organisms was not found, as reported for some bacteria (Tornabene et al., 1969). It was noted that the Analar light petroleum (nominally b.p. 85/100°C) used as the main organic component of the media was not completely free of hydrocarbons.
Cells were grown for various times in 250 ml flasks, each containing 100 ml YEME medium (A). After 44 h incubation, 10 g glucose 1-1 (sterile) was added to each of a group of 10 flasks (containing 10 g glucose 1-1 initially) and further incubated as shown in the table below (B). Cells were harvested by filtration from three flasks (300 ml) at the times indicated, and residual glucose in the bulked medium was determined. A, 10 g glucose 1-1 initially; B, plus 10 g glucose 1-1 after 44 h incubation; C, 20 g glucose 1-1 initially; ND, no actinorhodin detected (mycelium remained uncoloured). Experiments were done twice; the results presented are means of the two.

<table>
<thead>
<tr>
<th>Incubation period (h)</th>
<th>Residual glucose (g 1-1)</th>
<th>Actinorhodin (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>16</td>
<td>6-4</td>
<td>–</td>
</tr>
<tr>
<td>24</td>
<td>3-4</td>
<td>–</td>
</tr>
<tr>
<td>36</td>
<td>0-6</td>
<td>–</td>
</tr>
<tr>
<td>44</td>
<td>0-4</td>
<td>10-2</td>
</tr>
<tr>
<td>48</td>
<td>0-3</td>
<td>8-1</td>
</tr>
<tr>
<td>60</td>
<td>&lt; 0-1</td>
<td>5-1</td>
</tr>
<tr>
<td>68</td>
<td>&lt; 0-1</td>
<td>3-6</td>
</tr>
<tr>
<td>84</td>
<td>&lt; 0-1</td>
<td>1-8</td>
</tr>
</tbody>
</table>

40–60 °C or even 40–50 °C) used in the isolation procedure had to be distilled carefully to avoid contamination of concentrated fractions with saturated hydrocarbons. Small amounts of long-chain derivatives up to C₃₀ in chain-length (of the type CnH₂₃₊₂) were detected by mass spectroscopy of fractions obtained from concentration of redistilled solvent.

### Table 5. Synthesis of storage products by Streptomyces species

Organisms were grown in submerged culture in 250 ml flasks, each containing 100 ml YEME medium. Glycogen, polyhydroxybutyrate (PHB) and lipids were extracted from the mycelium formed by three flasks and assayed as described in the text. Results are the mean of duplicate determinations and are expressed per 1 medium. ND, < 0.1 g (glucose); < 0.1 mg (glycogen or polyhydroxybutyrate). Experiments were done twice and the results presented are means of the two.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Incubation period (h)</th>
<th>Residual glucose (g)</th>
<th>Dry weight (g)</th>
<th>Protein (mg)</th>
<th>Phospholipid (mg)</th>
<th>TAG (mg)</th>
<th>Glycogen (mg)</th>
<th>PHB (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. lividans</td>
<td>20</td>
<td>5-4</td>
<td>1-5</td>
<td>140</td>
<td>30</td>
<td>22</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>46</td>
<td>0-6</td>
<td>2-2</td>
<td>260</td>
<td>87</td>
<td>56</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>60</td>
<td>ND</td>
<td>3-0</td>
<td>300</td>
<td>90</td>
<td>126</td>
<td>80</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>S. coelicolor</td>
<td>20</td>
<td>4-7</td>
<td>1-4</td>
<td>140</td>
<td>29</td>
<td>26</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>46</td>
<td>0-6</td>
<td>2-1</td>
<td>280</td>
<td>88</td>
<td>100</td>
<td>50</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>60</td>
<td>ND</td>
<td>3-2</td>
<td>315</td>
<td>90</td>
<td>154</td>
<td>80</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>S. griseus</td>
<td>20</td>
<td>5-1</td>
<td>1-4</td>
<td>110</td>
<td>26</td>
<td>18</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>46</td>
<td>0-8</td>
<td>2-5</td>
<td>305</td>
<td>80</td>
<td>55</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>60</td>
<td>ND</td>
<td>3-6</td>
<td>320</td>
<td>84</td>
<td>92</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

### Relationship between TAG and polymer accumulation, and actinorhodin formation, in S. coelicolor

S. coelicolor was initially grown on complete medium but did not make actinorhodin under the conditions tested; glucose did not become exhausted from this medium and remained at a constant high level (see Table 2) during the late stages of growth. However, this organism did make pigment when grown on YEME medium but synthesis only commenced some 10 h or more after glucose exhaustion, which corresponded with maximum content of TAG in the mycelium. Continued pigment formation coincided with a drop in TAG levels at late incubation periods.

The effect of glucose on pigment synthesis was next examined more closely using S. coelicolor (J802). Cells grown in YEME medium containing glucose (10 g 1-1) served as control cultures (A). Other groups had additional glucose added at 44 h incubation (B) or contained initially 20 g 1-1 (C). All groups showed decreasing glucose content of the medium with time but only group A became exhausted with respect to glucose, at approximately 50–60 h (Table 4). Only mycelium derived from this group produced actinorhodin, bound as red pigment, but this feature was not observed until several hours after glucose had become exhausted from the medium. It was further noted in later experiments that occasionally some cultures did not make actinorhodin and that the time of onset was variable, but always considerably after the cells had entered stationary phase.

Further analysis was carried out to investigate the presence of other storage polymers (polyhydroxybutyrate and glycogen) in S. coelicolor, and other Streptomyces species for comparative purposes, which could potentially provide Cₘ units for metabolism and possible synthesis of...
produced polyhydroxybutyrate at any of the times tested with acetate-derived antibiotics. The results are presented in Table 5 and indicate that none of the organisms examined accumulated significant amounts of glycogen during the later stages of growth but not at earlier times. Very similar results were gained when the organisms were incubated in complete medium; only *S. lividans* formed glycogen. All the organisms studied produced TAG to similar extents on YEME (and also complete) medium (100–150 mg l\(^{-1}\) at 60 h), together with 80–90 mg phospholipid l\(^{-1}\) at this time. The pattern of TAG accumulation was similar to that earlier observed with complete medium but the mycelium took somewhat longer to reach stationary phase, at which point the protein and phospholipid content were rather higher (see Fig. 1 and Table 2 for comparison). Thus, TAG was the only storage product formed by *S. coelicolor* when grown on either medium.

**Table 6. Assay system for DAG acyltransferase**

The complete system used was as described in Methods and contained 100 µg protein derived from *S. lividans*, plus 30 µM DAG from *S. lividans* phospholipids (in 10 µl ethanol). Assays were carried out in duplicate. Lipids were extracted into hexane and were separated by TLC. Zones corresponding to TAG were scraped into scintillation vials and their radioactivity measured. Experiments were done twice; the results presented are means of the two.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Radioactivity in TAG (d.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>40340</td>
</tr>
<tr>
<td>Plus Tween 20 (1 mg ml(^{-1}))</td>
<td>10260</td>
</tr>
<tr>
<td>Enzyme omitted</td>
<td>300</td>
</tr>
<tr>
<td>Boiled enzyme</td>
<td>250</td>
</tr>
<tr>
<td>Zero time control</td>
<td>320</td>
</tr>
<tr>
<td>Minus DAG</td>
<td>35740</td>
</tr>
</tbody>
</table>

**Table 7. Effect of ethanol concentration on DAG acyltransferase activity**

Assays were performed in duplicate with 100 µg membrane protein from *S. lividans*, as described in Methods. No exogenous DAG was added to the system and ethanol volume was varied as indicated. TAG and alkyl ester were resolved from each other by TLC and appropriate zones were scraped off for determination of radioactivity. Experiments were done twice and the results presented are means of the two.

<table>
<thead>
<tr>
<th>Ethanol (µl)</th>
<th>Radioactivity incorporated (d.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TAG</td>
</tr>
<tr>
<td>0</td>
<td>11310</td>
</tr>
<tr>
<td>5</td>
<td>13050</td>
</tr>
<tr>
<td>10</td>
<td>12700</td>
</tr>
<tr>
<td>15</td>
<td>7040</td>
</tr>
<tr>
<td>20</td>
<td>5520</td>
</tr>
<tr>
<td>40</td>
<td>2560</td>
</tr>
</tbody>
</table>

Acidosis of polyhydroxybutyrate at any of the times tested with acetate-derived antibiotics. The results are presented in Table 5 and indicate that none of the organisms examined produced polyhydroxybutyrate at any of the times tested when grown in submerged culture on YEME medium. However, *S. lividans* (but not the other species) accumulated significant amounts of glycogen during the later stages of growth but not at earlier times. Very similar results were gained when the organisms were incubated in complete medium; only *S. lividans* formed glycogen. All the organisms studied produced TAG to similar extents on YEME (and also complete) medium (100–150 mg l\(^{-1}\) at 60 h), together with 80–90 mg phospholipid l\(^{-1}\) at this time. The pattern of TAG accumulation was similar to that earlier observed with complete medium but the mycelium took somewhat longer to reach stationary phase, at which point the protein and phospholipid content were rather higher (see Fig. 1 and Table 2 for comparison). Thus, TAG was the only storage product formed by *S. coelicolor* when grown on either medium.

**Variation in DAG acyltransferase activity with incubation period**

DAG acyltransferase catalyses the final step in TAG biosynthesis and is therefore unique to this pathway. This enzymic activity was first demonstrated in *S. lividans* and then in *S. coelicolor* and assayed as described in Methods. Activity was totally dependent on the presence of membrane protein (Table 6). The amount of labelled TAG produced was linear with increasing protein up to 400 µg during a 30 min assay period. TAG formation was also linear with time for 45 min when 100–200 µg protein was employed. Addition of Tween 20 severely inhibited formation of TAG (Table 6). A pH optimum of 8.5 was found, with activity dependent on the presence of enzyme at all pH values tested (7.0–9.0). Addition of ethanol (5–10 µl) to the reaction mixture gave a slightly enhanced activity but larger volumes of ethanol (15–40 µl) resulted in a decrease, with 80% inhibition noted when 40 µl of ethanol was added (Table 7). However, formation of alkyl ester was enhanced at higher concentrations. Addition of 10 µl methanol, 2-propanol or acetone each reduced activity by 60–70%. All results were similar for both organisms.

The results showed that reaction was often weakly dependent on added DAG (Table 6). In its absence, esterification generally proceeded at approximately 70% of the maximal rate shown in the presence of 1,2-dilaurin (30 µM added) or DAG obtained from *Streptomyces* phospholipids. Further addition of DAG (30 µM) halved the activity noted. 1,3-Dipalmitoylglycerol was ineffective, compared with the 1,2-isomer or 1,2-dilaurin, in stimulating activity. Attempts to remove lipid by solvent (acetone, methanol or ethanol) treatment, or by sonication or treatment with sodium cholate (50 mM), proved unsuccessful and resulted in severe reduction of enzyme activity, even on supplementation of the incubation system with dipalmitin. Significant relative activity in the absence of added DAG substrate remained, showing that solvent treatment had not removed all the endogenous DAG.

The distribution of DAG acyltransferase activity during growth of *S. coelicolor* on YEME medium is shown in Fig. 5; it paralleled the formation of TAG during growth. Activity was very low during the exponential phase, increasing considerably (approximately fivefold) as the cells entered the stationary phase (about 30 h). However, DAG acyltransferase activity dropped sharply (approx. 50%) in older cultures (45 h). Peak activity, expressed as either specific or total enzyme activity, appeared shortly after the onset of stationary phase. TAG levels, however, increased slightly at these times. These properties are similar to those reported for *S. lividans* grown on complete medium (Olukoshi & Packter, 1992), except that these...
cells reached stationary phase somewhat earlier and the transition from exponential to stationary phase was more clear cut.

Preliminary results have also established that a particulate TAG hydrolase (lipase) is present in the mycelium of *S. coelicolor* [red(−)] only during late stationary phase in YEME medium, at a time after glucose has become exhausted.

**DISCUSSION**

The relationship between growth of different species of *Streptomyces* and the synthesis of TAG and other lipids was fairly reproducible under various cultural conditions and examined at different incubation periods. Minimal formation of TAG was found during the exponential phase of growth but this lipid invariably accumulated during stationary phase, when net protein synthesis had ceased. This situation resulted in a relative increase in TAG production when *S. lividans* was transferred to a low-nitrogen or nitrogen-free medium. This condition would allow for the conversion of excess sugar into TAG, and indicates that the nitrogen content of the medium plays an important role in its accumulation. This aspect of metabolism resembles the synthesis of TAG in certain fungi and oleaginous yeasts (Ward & Packter, 1974; Ratledge, 1976; Holdsworth & Ratledge, 1988) and other storage polymers such as glycogen (Preiss & Romeo, 1989) and polyhydroxybutyrate (Dawes & Senior, 1973) in bacteria. However, TAG has not been considered a storage product in prokaryotes (Lennarz, 1966; Shaw, 1974), but *Streptomyces* do differ from many other bacteria in a number of features, not least in their filamentous nature and their ability to sporulate and to produce a wide range of antibiotics.

Among the species tested, only *S. lividans* formed glycogen. The most common energy reserve products in bacteria are glycogen, polyhydroxybutyrate and polyphosphate (Dawes, 1985; Preiss & Romeo, 1989), whereas TAG and glycogen predominate in eukaryotic cells (Dawes & Senior, 1973). Glycogen (and trehalose) have been found in aerial mycelium or spores of streptomycetes (Braña et al., 1986). Polysaccharides similar to glycogen have also been detected in the mycelium of various *Streptomyces* species when grown on the surface of cellophane membranes (Braña et al., 1982).

Significant amounts of isoprenoid hydrocarbons in the mycelium were produced by all the *Streptomyces* species examined, increasing somewhat during stationary phase, and this suggests that they may play a structural role in cell membranes (Kates, 1964; Tornabene et al., 1969). Presumably, they do not act as steroid precursors since sterols have not been found in *Streptomyces* or most other bacteria (Goldfine, 1982; Verma & Khuller, 1983), but polyisoprenoid derivatives (of longer chain-length) are involved as intermediates in the synthesis of peptidoglycan. Moreover, the increased synthesis of hydrocarbons during the stationary phase may be related to the ability of these organisms to sporulate in liquid culture. Streptomyces generally do not sporulate under these conditions (Chater & Merrick, 1979) but recent findings (Daza et al., 1989) have shown that a number of species, including *S. lividans*, may sporulate under appropriate nutritional (downshift) conditions. Hydrocarbons may further serve to provide a non-wettable surface surrounding the cell wall of the spores as is the case in fungal spores (Weete, 1980), plant surfaces (Kollatukudy, 1980) and insect cuticular surfaces (Jackson & Blomqvist, 1976; Weete, 1980).

TAG acyltransferase (TAG synthase) is an enzyme uniquely associated with the TAG biosynthetic pathway since all the other enzymes catalysing reactions from sn-glycerol 3-phosphate to DAG share reactions involved in phospholipid biosynthesis (Bell & Coleman, 1983; Murphy et al., 1991). Although the presence of this acyltransferase has been previously reported in *Escherichia coli*, the current work represents the first demonstration of this enzyme activity from *Streptomyces* spp., the only bacteria known to synthesize considerable amounts of TAG. Its distribution with incubation period for *S. coelicolor* (and also *S. lividans*) shows a strong correlation between activity, growth on various media and TAG content. The decrease in activity in older cultures (alongside an increase in TAG hydrolase) may simply reflect a change in the metabolic apparatus of the organism towards lipid utilization which would be needed at that time to maintain the integrity of the cells. A similar decrease in fatty acid synthase activity that corresponded to the onset of TAG utilization and phenol synthesis has been reported in *Aspergillus fumigatus* (Ward & Packter,

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*Fig. 5.* Variation in DAG acyltransferase activity during incubation of *S. coelicolor* (U802). Cells were grown in 250 ml flasks containing 100 ml YME medium for various time periods. Mycelium was harvested from two flasks and the results are expressed per 200 ml of medium. Enzyme was assayed as described in Methods and the activity determined based on endogenous DAG present in the membrane preparation. ●, Protein; ■, total radioactivity; □, specific radioactivity.
obtained from fat cells (Coleman S. 1974). The current studies have confirmed the production of large amounts of TAG from surplus glucose, as an energy store, which may be utilized later for various purposes when glucose has been exhausted from the medium. Synthesis and storage of TAG in Streptomyces may therefore be added to the list of unusual features of these bacteria.

In this study, the nature of the enzyme specificity towards different species of DAG was complicated by the presence of DAG (approaching saturating levels) bound to the membrane preparation used. This lipid was not readily removed by organic solvent or detergent treatment, which tended to inactivate the enzyme. A similar phenomenon has been reported in tissues such as chicken liver (Weiss et al., 1960) and cow mammary gland (Marshall & Knudsen, 1980).

Ethanol was found to be the best dispersing agent for DAG and when added at low concentration slightly stimulated enzyme activity. This property resembles the situation found for the mammalian enzyme (Goldman & Vagelos, 1961; Coleman & Bell, 1976; Marshall & Knudsen, 1980). Tween 20 has been used by other workers to disperse DAG samples (Sarzala et al., 1970; Van Golde et al., 1971) but it severely inhibited the acyltransferase in the Streptomyces preparations used.

A similar reduction in activity was reported for the enzyme obtained from fat cells (Coleman & Bell, 1976).

DAG acyltransferase activity was relatively stable when stored. Its pH optimum was similar to those reported previously for mammalian enzymes (Goldman & Vagelos, 1961; Van Golde et al., 1971; Coleman & Bell, 1976). The specific activity of the enzyme was similar in S. lividans and S. coelicolor and both activities were higher than that reported for E. coli (Pieringer et al., 1967). However, these activities are considerably lower than that found for mammalian enzyme (Coleman & Bell, 1976; Bell & Coleman, 1983). The distribution pattern of DAG acyltransferase activity during growth in both S. coelicolor and S. lividans indicates that this enzyme makes a major contribution to the de novo biosynthesis of TAG in these organisms, and it may be primarily responsible for the switch from membrane phospholipid formation to TAG biosynthesis which occurs during the stationary phase. Although a similar activity has been demonstrated in E. coli (Pieringer et al., 1967), its precise role has been questioned (Pieringer, 1983). The small amounts of DAG found in this organism (less than 1% of total lipid) (Raetz & Newman, 1979) may be artifactual, arising from the extraction procedure (Pieringer, 1983; Kates, 1988). Metabolically, DAG has been regarded a dead-end technology. In this organism (less than 1% of total lipid) (Raetz & Newman, 1979) may be artifactual, arising from the extraction procedure (Pieringer, 1983; Kates, 1988). Metabolically, DAG has been regarded a dead-end

PACKTER, 1992). Certainly in S. coelicolor, TAG appears to be a possible source of acetyl-CoA for this purpose since neither glycogen nor polyhydroxybutyrate was detected. Indeed, polyhydroxybutyrate was not found in any of the Streptomyces spp. examined. Kannan & Rehacek (1970) suggested that polyhydroxybutyrate might serve as the source of C₂ units for antibiotic synthesis but its presence in mycelium was not confirmed in the current work. Glucose itself, usually a suitable carbon source for growth, has been shown to repress the formation of a wide variety of antibiotics in Streptomyces (Martin & Demain, 1980; Vining & Doull, 1988; Bushell, 1988). Indeed, Hobbs et al. (1990) showed that cultures of S. coelicolor produced actinorhodin, an acetate-derived antibiotic, only when glucose had been exhausted from the minimal medium used. This phenomenon has been confirmed in the present studies, which also showed that ‘surplus’ glucose when added initially, or even during late stationary phase, prevented actinorhodin formation.

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REFERENCES


Stored triacylglycerols in *Streptomyces*


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