Arch Microbiol (1995) 164:420–427

ORIGINAL PAPER

Neville M. Packter · Ekundayo R. Olukoshi

Ultrastructural studies of neutral lipid localisation in *Streptomyces*

Received: 6 June 1995 / Accepted: 4 September 1995

Abstract Triacylglycerol is accumulated by *Streptomyces* spp. when grown in submerged culture. Ultrastructural studies using transmission electron microscopy (TEM), staining and freeze-fracture/freeze-etch procedures, and light microscopy confirmed the accumulation of neutral lipid by *S. lividans* and *S. coelicolor* during the stationary phase and its storage within membrane-bound globular structures within the cytoplasm. These structures were of various sizes and occupied up to approximately 80% of the total cell volume at that time. There was no evidence of such material within cells examined during the early exponential phase of growth. The globules visualised by TEM were electron-transparent since they comprised lipids containing saturated fatty acids that did not react with osmium tetroxide. The globules appeared to be bounded by a single membrane.

Key words Transmission electron microscopy · Light microscopy · Triacylglycerols · *Streptomyces* · Sudan black B

Abbreviations *TAG* triacylglycerol · *TEM* transmission electron microscopy

Introduction

Members of the genus *Streptomyces* are filamentous gram-positive bacteria characterised by unusual morphological complexity and extreme versatility in making a variety of secondary metabolites, many of which are capable of antibiotic activity (Martin and Demain 1980; Daza et al. 1989) and are in clinical use. Another unusual feature of these bacteria is their ability to sporulate. Indeed, all these unusual characteristics may be involved in the survival strategy of *Streptomyces* bacteria (Chater and Merrick 1979).

We have recently shown that *Streptomyces* spp., perhaps uniquely among prokaryotes, accumulate considerable amounts of triacylglycerol (TAG; 50–150 mg l^{-1} of medium) at late stages of submerged growth (Olukoshi and Packter 1994). This neutral lipid is an atypical energy reserve compound in bacteria, whereas glycogen, polyhydroxybutyrate and polyphosphates frequently function in this capacity (Dawes 1985). In addition, it has been demonstrated in *S. coelicolor* that glucose exhaustion is accompanied by a levelling off in TAG levels within the mycelium and that this event is followed by antibiotic formation. It has been suggested that TAG might act as the carbon source for those antibiotics derived from acetyl-CoA/malonyl-CoA precursors (Olukoshi and Packter 1994).

This paper presents further insight into the accumulation of TAG in *Streptomyces* after examining its location within the mycelium (mainly using *S. lividans*) by means of ultrastructural studies involving electron and light microscopy. These studies highlight some of the difficulties in fixing and staining cells and in post-staining sections of *Streptomyces* cells by reaction with osmium tetroxide, caused by the absence of unsaturated fatty acyl residues in their lipids.

Materials and methods

Source of materials

General chemicals and reagents for microscopy were obtained from BDH Chemicals. Agar and other media components were purchased from Lab M Products.

Culture conditions

Streptomyces lividans 66 and *S. coelicolor* A3(2), an agarase-minus mutant of the wild type, were obtained originally from the culture collection at the John Innes Institute, Norwich, UK by cour-

N. M Packter (✉) · E. R. Olukoshi
Department of Biochemistry and Molecular Biology,
The University of Leeds, Leeds LS2 9JT, UK
Tel. +44-1132-333150; Fax +44-1132-333167
e-mail n.m.packter@leeds.ac.uk

tesy of Prof. D.A. Hopwood. Stock cultures were maintained on agar slants. Spores were obtained as described by Olukoshi and Packter (1994) and used to inoculate complete medium (Hopwood 1967). *S. lividans* was generally used for these studies because it produced greater yields of TAG than other species and, moreover, did not form pigment in liquid culture. Cells were incubated in 250-ml Erlenmeyer flasks containing 100 ml medium on an orbital shaker (250 rpm, 30° C) for various periods corresponding to early exponential phase and to early and late stationary phase of growth (Olukoshi and Packter 1994).

Transmission electron microscopy (TEM) staining

Cells were harvested by centrifugation at 4000 × *g* for 5 min and the mycelium from 20 ml culture medium was washed three times with 20 ml phosphate buffer held at 2° C (0.1 M, pH 7.4). Cells were fixed by reaction with glutaraldehyde (2.5%, v/v in 0.1 M phosphate buffer, pH 7.4) for 4 h at 4° C and washed three times at 4° C for 2 h in this buffer. Cells were then treated with osmium tetroxide (0.1%, w/v in 0.1 M phosphate buffer) at 4° C for 2 h (Ryter and Kellenberger 1958) and washed in this buffer at 4° C for 15 min. Cells were dehydrated in a graded ethanol series at 4° C (to reduce loss of lipids) as follows: 70% ethanol (2 × 10 min each), 90% ethanol (2 × 10 min each) and 100% ethanol (3 × 20 min each). They were stained initially in this first step with a low concentration of uranyl acetate (4%, w/v in 70% ethanol) for 1 h. The dehydrated cells were embedded in Epon 812 resin by transferring them into propylene oxide (2 × 10 min each) and exchanging to a mixture of propylene oxide and resin (1:1) for 6 h. Cells were then placed in 100% resin for 24 h under vacuum, leaving the cap off to allow for solvent evaporation. Finally, cells were blocked out in fresh resin and allowed to polymerise at 60° C for 48 h. Blocks were cut on a Reichert Ultracut ultramicrotome with glass knives, and sections were picked on copper grids and re-stained for 5 min each with uranyl acetate (15%, w/v in methanol) and lead citrate (Reynolds 1962) prior to TEM. Due to the mycelial nature of the cells, sections of variable thickness ranging from 50 nm to, more usually, 100 nm were obtained.

Although the filamentous nature of the cells and their thick cell wall made it difficult to section them uniformly, it was important to obtain thin sections for examination. Moreover, modified staining procedures (initial staining during the first step of the dehydration process and before blocking) helped to enhance contrast. The sections were examined in a JEOL 100S transmission electron microscope at 100 kV.

Freeze-fracture/freeze-etch procedures

Mycelium was harvested and fixed with glutaraldehyde as described above and washed four times (20 min each) with this reagent. Cells were subsequently cryoprotected with glycerol (30%, v/v in 0.1 M phosphate buffer). Tissue was frozen in liquid nitrogen slush on gold/nickel supports and stored under liquid nitrogen until further analysed. Specimens were fractured at 163°K or etched for 2 min at 173°K in a Balzers BAF 400D freeze-fracture unit. Platinum/carbon replicas were laid down from 45° (Pt/C layer approximately 20Å; C layer approximately 200Å). Replicas were floated clear of bulk sample and then transferred to distilled water prior to cleaning with 40% (w/v) NaOCl. Cleaned replicas were picked up on pioloform-coated 200 mesh copper grids, examined and photographed using a Philips CM10 electron microscope at 60 kV.

Light microscopy: Sudan black B stain

Staining with Sudan black B was carried out as described by Burdon (1946). A thin film of mycelium was prepared and air-dried for 30 min before fixing by heat treatment. The entire slide was flooded with Sudan black B (0.3%, w/v in 70% ethanol) and left undisturbed at room temperature for 30 min. The excess stain was drained off and the slide blotted dry. The slide was treated by dropwise addition of ethanol and blotted dry before counterstaining with safranine (0.5%, w/v in water) for 10 s; then it was washed in water and air-dried. Specimens were examined on a Nikon Optophot microscope using an oil immersion lens. Mycelium containing neutral lipid appeared deep purple, whereas the safranine counterstain gave rise to a pink colour in cells that did not contain triacylglycerol.

Results

Localisation of TAG within cells

A series of electron and light microscopic studies was performed to localise the TAG accumulated in cells of *Streptomyces*. Accordingly, *S. lividans* was grown in complete medium and cells were harvested at three stages of

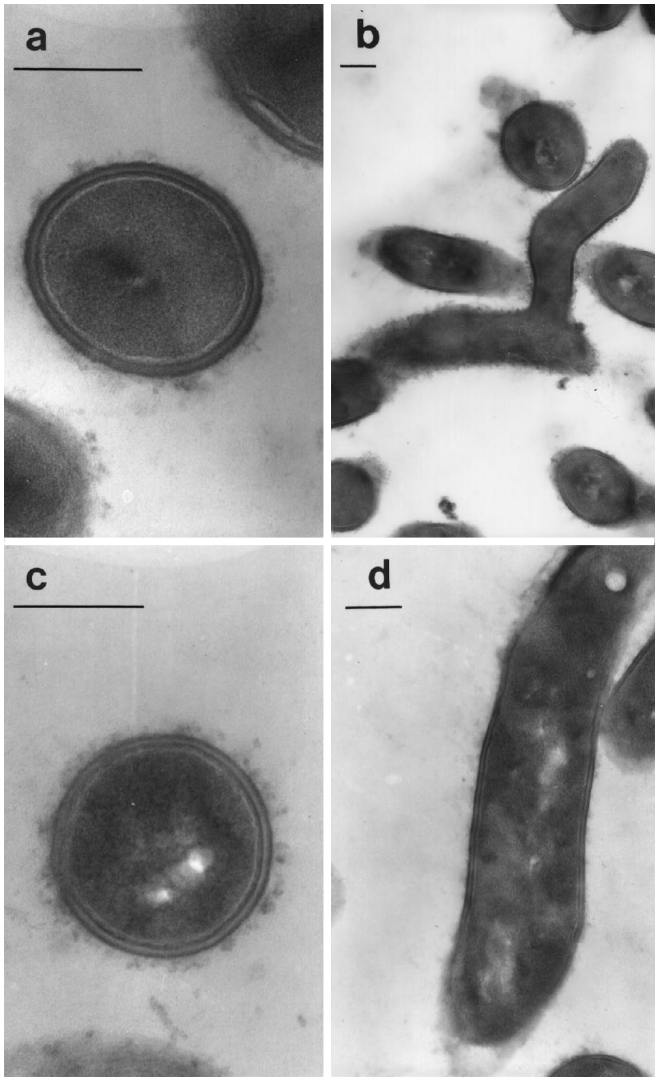

Fig. 1a–d Transmission electron micrographs of sections through cells of *Streptomyces lividans*. Mycelium was harvested by centrifugation after growth for 12 h (**a, b**) and 24 h (**c, d**) in submerged conditions on complete medium. It was washed with 0.1 M sodium phosphate buffer, prefixed with glutaraldehyde and postfixed as described in Materials and methods. Sections were examined in a JEOL 100S transmission electron microscope (*bar* 0.2 μm)

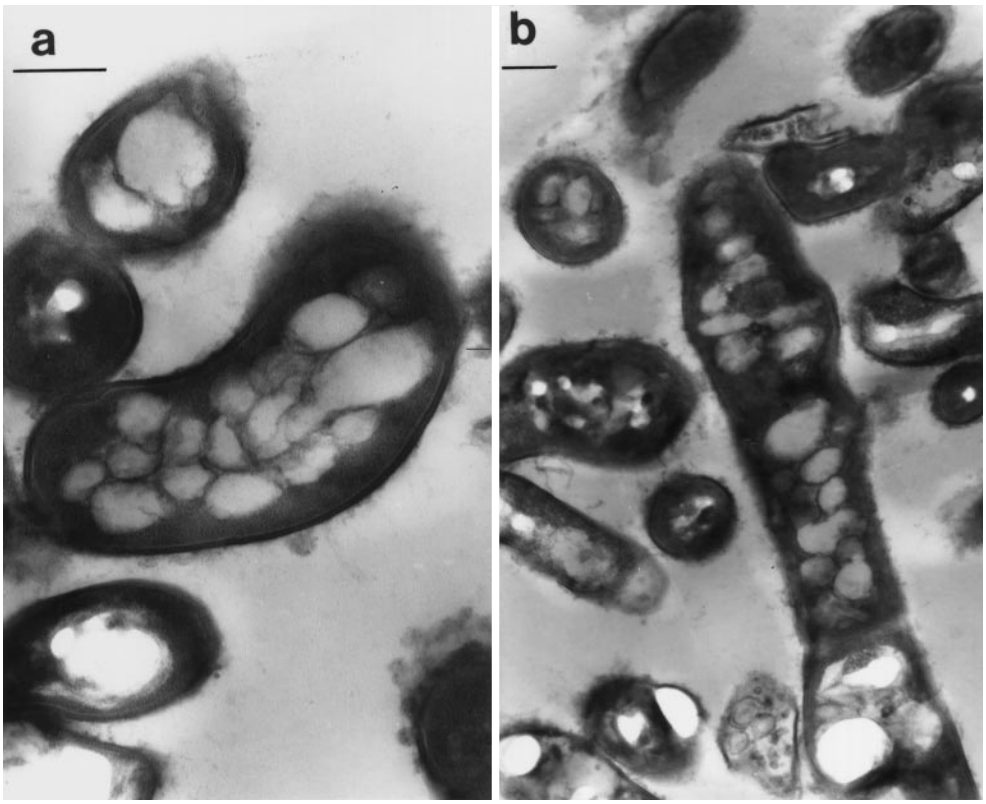

Fig. 2a, b Transmission electron micrographs of sections of *Streptomyces lividans* during late stationary phase. Cells were incubated for 48 h (**a, b**) and prepared for electron microscopy as described in Fig. 1 (*bar* 0.2 μm)

growth: 12 h, 24 h and 48 h. These incubation periods corresponded to early exponential phase (absence, or very low content, of TAG), early stationary phase (onset of TAG accumulation) and late stationary phase (period of maximum accumulation), respectively. At these times, approximately 0, 50 and 125 mg TAG were present in mycelium per litre of medium (Olukoshi and Packter 1994).

Tranmission electron microscopy

Since the fatty acid residues in *Streptomyces* lipids are all saturated (Ballio et al. 1965; Packter et al. 1985), these would not react with osmium tetroxide and stain positively for the TEM studies. It was, therefore, important to obtain thin sections and to apply modified procedures in the treatment with heavy metal salts to maximise contrast arising from the negative staining effect (see Materials and methods).

Electron micrographs from thin sections of *S. lividans* cells at different stages of growth and stained with these salts are shown in Figs. 1 and 2. After 12 h incubation (Fig. 1a, b), when TAG was present in very small amounts (and probably absent from most cells), the cytoplasm was devoid of visible internal structures and had a characteristic granular appearance. A similar pattern was observed in sections obtained from cells grown for 24 h (Fig. 1c, d), except that one or two electron-transparent regions were usually present. Cells at this stage had begun to accumulate TAG (Olukoshi and Packter 1994). However, sections of 48-h mycelium (Fig. 2a, b) showed an abundance of electron-transparent vesicles within the cytoplasm that had no internal structure. These discrete structures appeared to be bordered by a thin membrane, were of variable sizes and occupied a substantial portion of the total cell volume (approximately 70–80%). The width of this membrane (3–4 nm) was approximately half the size of the cell membrane (Fig. 3). No structures were detected alongside this membrane that might have corresponded to enzymes involved in lipid synthesis. Very similar micrographs were obtained when sections from *S. coelicolor* were examined after comparable stages of growth.

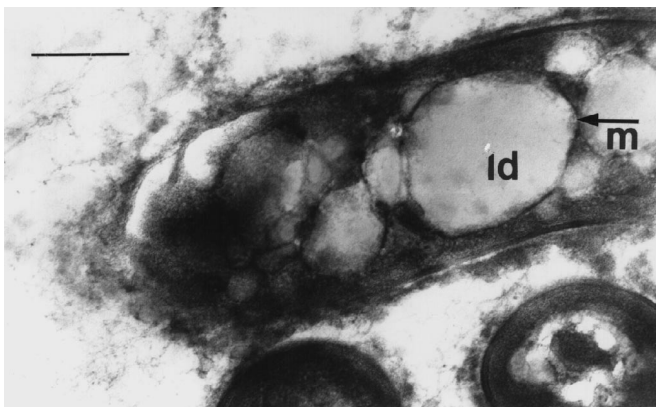

Fig. 3 Portion of a cell of *Streptomyces lividans* (48 h) showing the membrane *m* surrounding the lipid droplets *ld*. Cells were prepared for electron microscopy as described in Fig. 1 (*bar* 0.2 μm)

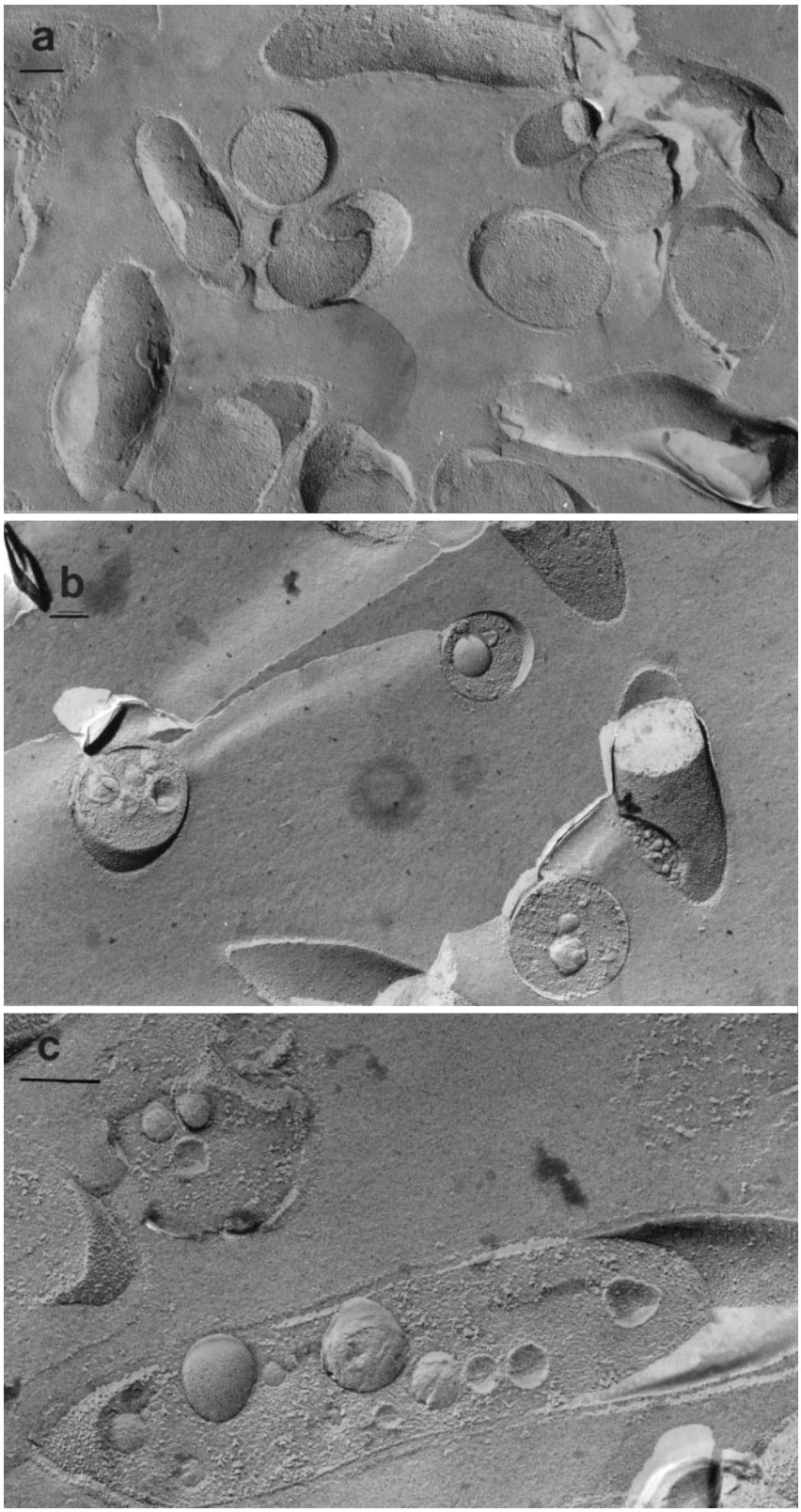

Fig. 4a–c Micrographs of freeze-fractured cells of *Streptomyces lividans*. This organism was incubated in complete medium for **a** 12 h, **b** 24 h and **c** 48 h, and mycelium was collected, washed and prefixed in glutaraldehyde as described in Fig. 1. Cells were frozen in liquid nitrogen and fractured. Replicas were picked on piloloform-coated copper grids and examined in a Philips CM10 electron microscope (*bar* 0.2 μm)

Freeze-fracture/freeze-etch procedures

Further studies were carried out using these procedures and with cells grown for similar periods as in the studies described above. The patterns observed with freeze-fractured cells (Fig. 4) and freeze-etched cells (Fig. 5) were similar to those obtained with heavy-metal, postfixed cells in that only 48-h sections showed an abundance of cytoplasmic

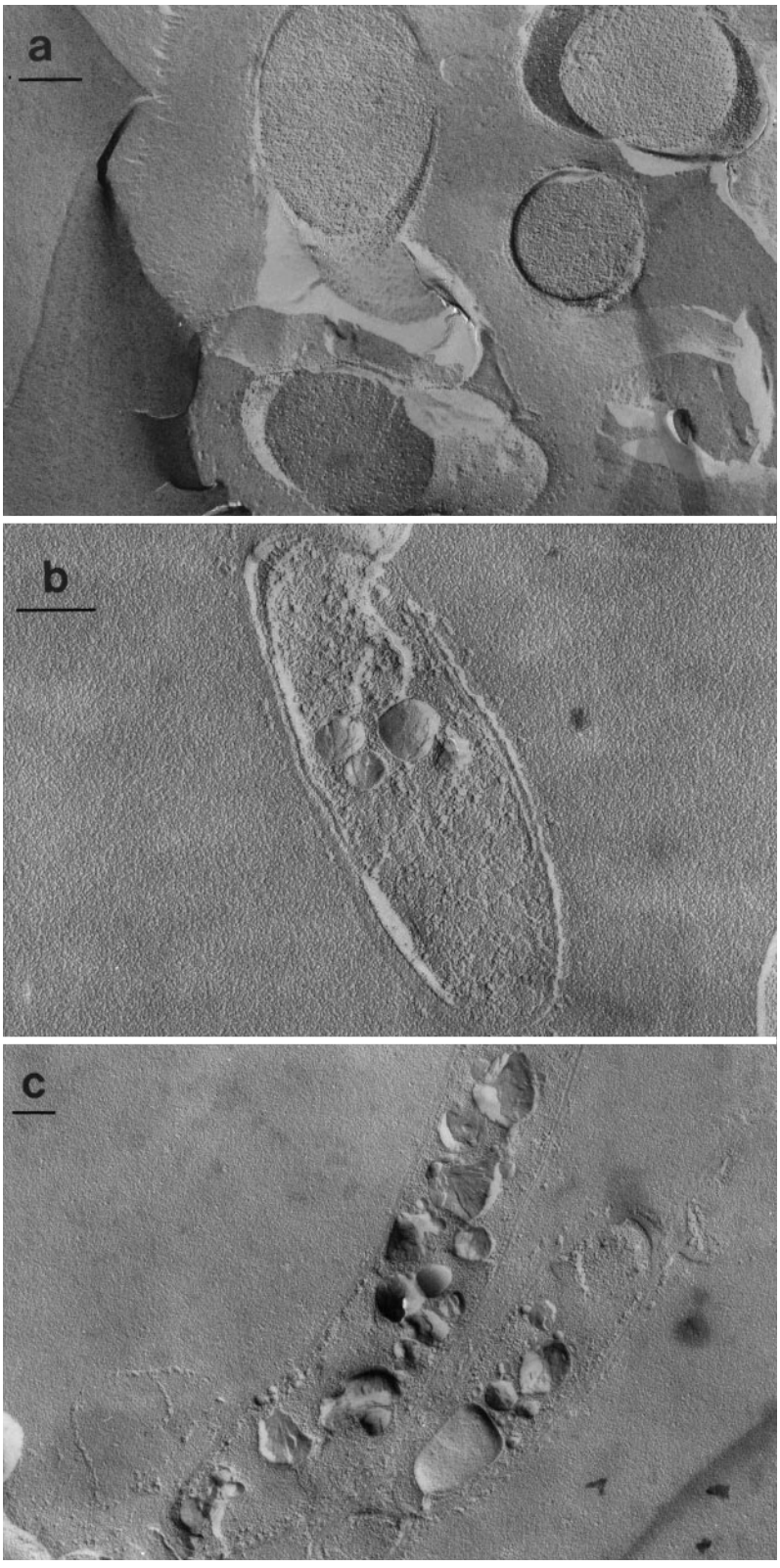

Fig. 5a–c Micrographs of freeze-etched cells of *Streptomyces lividans*. Cells were obtained after various incubation periods as described in Fig. 3. They were frozen under a slush of liquid nitrogen and freeze-etched at –173° K. Replicas were obtained as described in Materials and methods. Incubation periods were **a** 12 h; **b** 24 h; **c** 48 h (*bar* 0.2 μm)

globular structures. Sections obtained at 12-h incubation were devoid of any internal features (Figs. 4a, 5a), while cells grown for 24 h contained only a few globular structures. These structures showed wide variations in size, but in 48-h cells, they occupied large sections of the cytoplasm within individual cells of the mycelium (Fig. 4c, 5c). 'Peaks' and 'troughs' could be clearly seen (Fig. 4c) depending on which aspect of the fractured face was viewed.

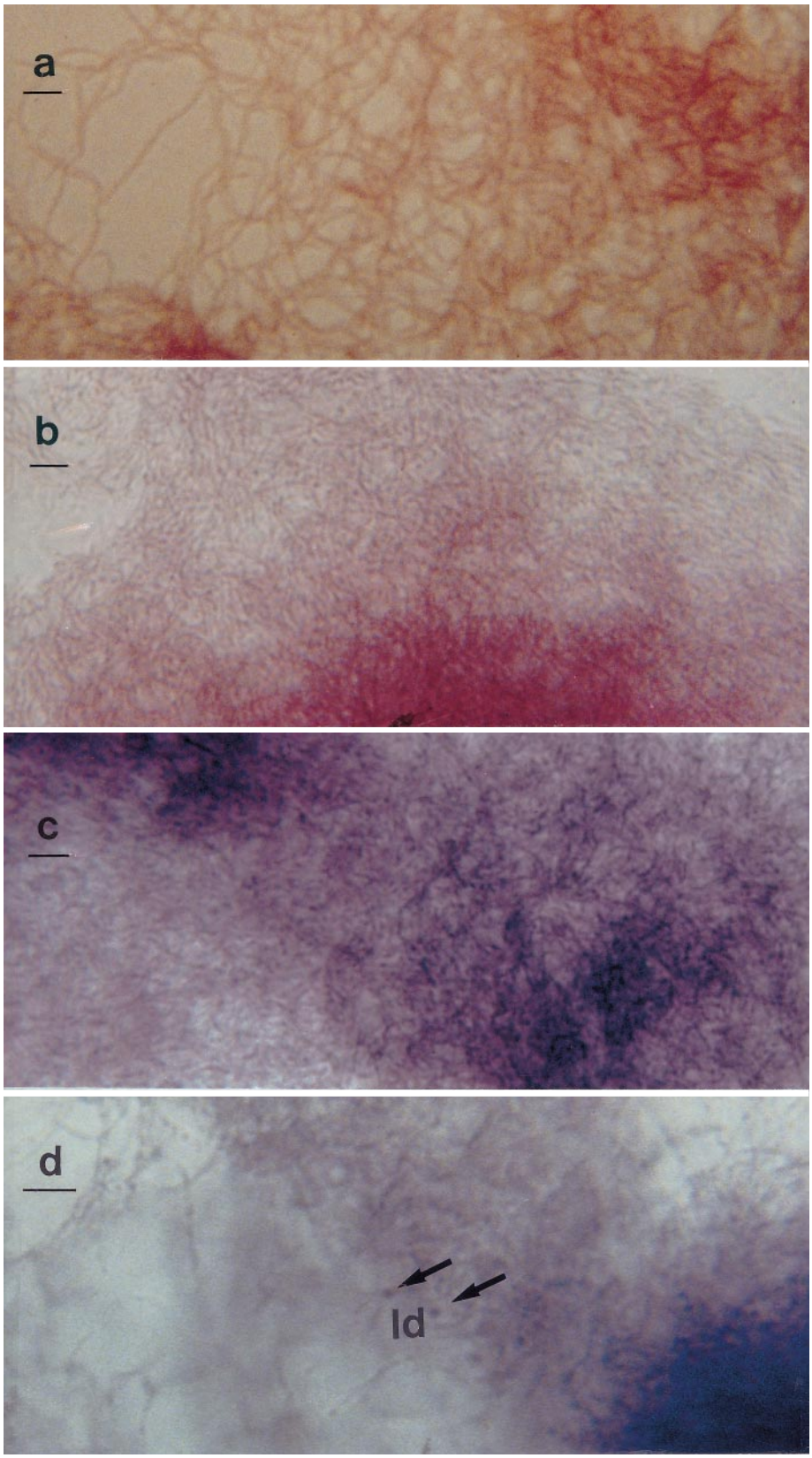

Fig. 6a–d Photomicrographs of *Streptomyces lividans.* Cells were incubated for **a** 12 h, **b** 24 h, **c** and **d** 48 h in complete medium. Lipid droplets *ld* are shown in **d**. Cells were stained with Sudan black B, counterstained with safranine and examined under oil immersion (*bar* 10 μm)

Light microscopy

Light microscopy studies in which cells were stained with Sudan black B were carried out to confirm the lipid nature of these inclusions. This dye is an intracellular stain that reacts specifically with neutral lipids, e.g. triacylglycerol, giving a characteristic purple colour (Burdon 1946). Using this dye, cells incubated for 12 h at an early exponential phase of growth (Fig. 6a) had an overall pink appearance corresponding only to the colour of the safranine counterstain. Somewhat older cultures (24 h) had a red-purplish appearance (Fig. 6b); cells at this stage of growth were just beginning to accumulate neutral lipid. However, cells obtained at later stages of incubation and well into the stationary phase (48 h) had an overall dark purple appearance, indicating the presence of large amounts of TAG (Fig. 6c). A careful examination of the hyphae at the edge of colonies revealed the presence of numerous tiny droplets along their entire length. These were especially visible in the purple-stained 48-h cells (Fig. 6d).

Discussion

Ultrastructural studies involving certain techniques of sample preparation, such as ultrathin sectioning, staining and freeze-fracture/etch procedures, followed by TEM and light microscopy clearly confirmed the accumulation of lipid by cells of *S. lividans* during the stationary phase and its storage within globular structures in the cytoplasm. It has been previously established that TAG is formed only in small amounts during the exponential phase in various *Streptomyces* spp. Synthesis increases during the stationary phase, and the amount present is maintained until at least 50–60 h, when TAG accounts for approximately 60% of the total cellular lipids (Olukoshi and Packter 1994).

The globular structures reported in this paper presumably corresponded to the accumulation of TAG droplets which had not become stained, and they appeared to be membrane-bound with little tendency to coalesce. The presence of this membrane and its apparent size were very similar to those found in bacterial cells that accumulate polyhydroxybutyrate. This storage polymer also appears transparent after the staining procedures, and the granules containing it are limited by a membrane approximately 2–4 nm thick (Jensen and Sicko 1971; Shively 1974). A similar ‘single bounding line’ has been found surrounding oil (TAG) droplets in *Crambe* seed cells (Gurr et al. 1974).

The droplets showed wide variations in size and, in later cultures, occupied the bulk of the cytoplasm. The freeze-etching/fracturing procedures clearly indicated regions within the cell that had cleaved during these processes and supported the nature of membrane-bound droplets. These procedures did not include dehydration using ethanol and, therefore, permitted the retention of lipid within the cells that had been fixed. Presumably, the fracturing occurred along weaknesses related to structural inhomogeneities alongside the membrane/cytoplasm interface rather than at cleavage planes within a bilayer membrane.

The neutral lipid nature of these cytoplasmic inclusions was confirmed by treatment with Sudan black B. Although the presence of lipid inclusion bodies has been noted in the cytoplasm of *Mycobacterium smegmatis* (Gale and McLain 1963), the nature of these bodies was not established, and studies relating specifically to the localisation of TAG had not previously been performed on bacteria. This lipid was readily identified in the current investigations by the strong response to the stain used, especially during the late stationary phase. No trace of purple colour was seen in cells isolated at the early exponential phase because the cells did not contain TAG. This neutral lipid is also stored as discrete droplets of varying sizes in the cytoplasm of oil seeds (Gurr et al. 1974; Gurr 1980) and oleaginous yeasts (Holdsworth et al. 1988). However, in animals TAG is stored as a single, large droplet in the cytosol of the adipocyte (Gurr and Harwood 1991).

The electron-transparent nature of the oil-bodies detected in the current studies is due to a lack of specific staining with osmium tetroxide, which is normally used to stain lipids of eukaryotic cells (Friend 1969; Gurr et al. 1974) This reagent cross-links specifically with double bonds in the unsaturated fatty acyl residues of lipids present in most cells to generate an electron-dense product, and it does not react with saturated fatty acids (Hayat 1989). Fatty acids present in the acyl lipids of *Streptomyces* are saturated (Olukoshi and Packter 1994); they correspond predominantly to the branched-chain of the *iso*- and *anteiso*- series (Ballio et al. 1965; Packter et al. 1985) and would not respond positively to this stain. The structures visualised arose effectively through a negative staining effect, although osmium tetroxide [osmium (VIII) reagent] does react with some amino acid residues, particularly cysteine, methionine and tryptophan (Deetz and Behrman 1981). It would, therefore, stain proteins in the cell membrane and in any internal membrane surrounding cytoplasmic inclusions.

Droplets have been noted previously in electron micrographs of *Streptomyces* species, but these were described simply as vacuoles (Wildermuth and Hopwood 1970) or attributed to the presence of polyhydroxybutyrate (Chater and Merrick 1979). This polymer, however, is present only in small amounts (1–15 mg/l of culture in various species; Kannan and Rehaceck 1970; Ranade and Vining 1993) and could not be detected when some species were examined (Olukoshi and Packter 1994).

Glycogen and trehalose have also been found in aerial mycelium and maturating spores in *S. antibioticus* when grown on cellophane membranes placed on solidified medium (Braña et al. 1982, 1986), but these carbohydrates appeared in micrographs as granules (rather than as membrane-bound ‘vacuoles’) after treatment with periodic acid and suitable reagents. Moreover, trehalose was not detected in non-sporulating, vegetative cultures of *S. venezuelae* but was present in cells grown in medium that

supported sporulation and in spores formed on agar (Ranade and Vining 1993).

Acknowledgements We thank the Commonwealth Scholarship Commission, UK, for the award of a research scholarship to E. R. Olukoshi. We also thank Paul McPhie and Denise Ashworth for expert technical assistance in electron microscopy.

References

Ballio A, Barcellona S, Boniforti L (1965) The component fatty acids of lipids from some *Streptomyces* species. Biochem J 94:11C–13C

Braña AF, Manzanal MB, Hardisson C (1982) Characterization of intracellular polysaccharides of *Streptomyces*. Can J Microbiol 28:1320–1323

Braña AF, Méndez C, Díaz LA, Manzanal MB, Hardisson C (1986) Glycogen and trehalose accumulation during colony development in *Streptomyces antibioticus*. J Gen Microbiol 132: 1319–1326

Burdon KL (1946) Fatty material in bacteria and fungi revealed by staining dried, fixed slide preparations. J Bacteriol 52:665–678

Chater KF, Merrick MJ (1979) Streptomycetes. In: Parish JH (ed) Developmental biology of prokaryotes. University of California Press, Berkeley Los Angeles, pp 93–114

Dawes (1985) Starvation, survival and energy reserves. In: Fletcher M, Floodgate GD (eds) Bacteria in their natural environments. Society of General Microbiolgy Special Publication, no 16. Academic Press, London, pp 43–79

Daza A, Martin JF, Dominguez A, Gil JA (1989) Sporulation of several species of *Streptomyces* in submerged cultures after nutritional downshift. J Gen Microbiol 235:2483–2491

Deetz JS, Behrman EJ (1981) Reaction of osmium reagents with amino acids and proteins. Int J Pept Protein Res 17:495–500

Friend DS (1969) Cytochemical staining of multivesicular body and Golgi vesicles. J Cell Biol 41:269–279

Gale GR, McLlain HH (1963) Effect of ethambutol on cytology of *Mycobacterium smegmatis*. J Bacteriol 86:749–756

Gurr MI (1980) The biosynthesis of triacylglycerols. In: Stumpf PK (ed) The biochemistry of plants, vol 4. Academic Press, New York London, pp 205–249

Gurr MI, Harwood JL (1991) Lipids as energy stores. In: Lipid biochemistry, 4th edn. Chapman & Hall, London, pp 126–130

Gurr MI, Blades J, Appleby RS, Smith CG, Robinson MP, Nichols BW (1974) Triglyceride biosynthesis and storage in whole seeds and oil bodies of *Crambe abyssinica*. Eur J Biochem 43:281–290

Hayat MA (1989) Chemical fixation. In: Principles and techiques of electron microscopy: biological applications, 3rd edn. Macmillan, London, pp 42–46

Holdsworth JE, Veenhuis M, Ratledge C (1988) Enzyme activities in oleaginous yeasts accumulating and utilizing exogenous or endogenous lipids. J Gen Microbiol 134:2907–2915

Hopwood DA (1967) Genetic analysis and genome structure in *Streptomyces coelicolor*. Bacteriol Rev 31:373–403

Jensen TE, Sicko LM (1971) Fine structure of poly-β-hydroxybutyric acid granules in a blue-green alga, *Chlorogloea fritschii*. J Bacteriol 106:683–686

Kannan LV, Rehacek Z (1970) Formation of poly-β-hydroxybutyrate by Actinomycetes. Indian J Biochem 7:126–129

Martin JF, Demain AL (1980) Control of antibiotic synthesis. Microbiol Rev 44:230–251

Olukoshi ER, Packter NM (1994) Importance of stored triacylglycerols in *Streptomyces*: possible carbon source for antibiotics. Microbiology 140:931–943

Packter NM, Flatman S, Lucock AJ (1985) Formation of storage lipids and actinorhodin, a phenolic antibiotic in *Streptomyces coelicolor*. Biochem Soc Trans 13:251–252

Ranade N, Vining LC (1993) Accumulation of intracellular carbon reserves in relation to chloramphenicol biosynthesis by *Streptomyces venezuelae*. Can J Microbiol 39:377–383

Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208–212

Ryter A, Kellenberger E (1958) Étude au microscope électronique de plasmas contenant de l'acide désoxyribonucleique. Z Naturforsch 13B:597–605

Shively JM (1974) Annu Rev Microbiol 28:167–187

Wildermuth H, Hopwood DA (1970) Septation during sporulation in *Streptomyces coelicolor*. J Gen Microbiol 60:51–59