Morphological and Chemical Nature of the Sclerotia of *Chainia olivacea* Thirumalachar and Sukapure of the Order *Actinomycetales*

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The biochemical properties of strains of most of the known species of *Chainia* Thirumalachar were compared. The mode of formation of sclerotia by a strain of *C. olivacea* Thirumalachar and Sukapure was studied by light and electron microscopy. Hyphae first show numerous lipid inclusions and then divide into cells in which lipid occupies more and more space. An intercellular cement is deposited forming a plurilocular structure. The lipids, which account for 50% of the weight of the biomass, are triglycerides of branched-chain fatty acids of the iso/anteiso series, principally C₁₅ and C₁₆. The intercellular cement is rich in L-2,3-diaminopropionic acid, an amino acid not previously found associated with microbial structures.

The genus *Chainia* was proposed by Thirumalachar in 1955 (38). The description of the new genus was as follows: "Mycelium well developed, fine, non-septate, growing into substrate, not forming true aerial mycelium, non-fragmenting, Gram-positive, non-acid fast, developing sclerotic granules composed of incurled hyphal cells, single or in aggregates, enveloped in hyphal sheath at first and later becoming free. Conidial form unknown. Type species '*Chainia antibiotica* Thirum'."

Gattani (13) observed the production of sclerotic granules by two strains of *Streptomyces griseus* grown on Trypticase soy agar. The granules were initiated by the swelling of a hypha which became surrounded by others leading to formation of granules 12 to 45 µm in diameter which could coalesce into small groups.

After the publication of the description of *C. antibiotica* in 1955, Thirumalachar distributed strains of this organism together with strains of two undescribed species, *C. poonensis* and *C. olivacea*. The latter was eventually described by Thirumalachar and Sukapure in 1964 (40). *C. poonensis* was never described by Thirumalachar, but a description of this organism was published by Kalakoutskii and Krasil'nikov in 1960 (22). Additional species were described as follows: *C. violens* (22), *C. ochracea* (24), *C. flava*, *C. purpurogena* (40), *C. minutisclerotica* (39), *C. rosea*, *C. fumigata*, and *C. aurea* (41).

Two other species of Thirumalachar, *C. nigra* and *C. rubra*, were described by Shirling and Gottlieb (35).

It soon became obvious to those who examined cultures of *Chainia* that the original description by Thirumalachar was misleading since strains of *Chainia* formed aerial mycelium bearing chains of conidia identical to those formed by strains of *Streptomyces*. In addition, strains of *Chainia* were found to have a cell wall composition identical to that of streptomycetes (4). Definition of the genus *Chainia* on the basis of production of sclerotia became difficult to accept especially since Baldacci et al. (2) confirmed Gattani's report that a large number of actinomycetes can form sclerotic granules.

The aim of the present study is to follow the mode of sclerotium formation in a strain of *C. olivacea*, to compare the chemical composition of the wall of the sclerotium with that of the hyphae of this organism, and to gain some knowledge about the nature of the lipids formed in the sclerotium.

MATERIALS AND METHODS

Organisms. *Chainia olivacea* strain V17676 was isolated from nondesertic soil by Enrique Tejera, National Institute of Hygiene, Caracas, Venezuela. All the other strains of *Chainia* reported here were our own soil isolates or were received from M. J. Thirumalachar or R. S. Sukapure of Hindustan Antibiotics Ltd., Pimpri (Nr. Poona), India. Strain numbers are those of the collection of the Institute of Microbiology of Rutgers University (IMRU) if not otherwise indicated.

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Growth and maintenance of cultures. Cultures were grown either from a freeze-dried stock or from stocks maintained on slants. All strains were maintained on yeast extract-dextrose (YD) agar slants (yeast extract, 10 g; crude dextrose, 10 g; agar, 15 g; tap water, 1 liter, pH 7.2 before sterilization) grown at 28 C for 7 to 10 days. Sclerotia for chemical analyses were harvested by scraping 1-week-old growth on plates of Czapek (Cz) agar (44) using the edge of a microscope slide or a scalpel. Vegetative cells used in the preparation of cell walls were grown for one week in Czapek broth on a reciprocal shaker (New Brunswick Scientific Co., New Brunswick, N. J., model 5713 operated at 60 strokes/min with a stroke length of 20 cm).

Other media used for morphological studies were potato-carrot (PC; 10), dilute potato-carrot (TPC), and water agar (WA) (21) made with either crude or Noble (Difco) agar and dilute Pabulum (TPab; 29).

Physiology. The methods used were those of Gordon (16) and Gordon and Horan (17).

Whole-cell analysis. The procedures used were those previously described (27).

Cell wall preparation. Preparations and analyses of cell walls and sclerotial walls were made using the method described by Šuput et al. (37) except that various methods of breakage had to be used. Vegetative cells of the substrain of V17676 which had lost the capacity to form sclerotia could be broken (like most actinomycetes) by sonic treatment for 10 min. The vegetative cells of the substrain still capable of forming sclerotia withstood 30 min of sonic treatment and 30 min of grinding in a tissue grinder. Breakage was finally accomplished by grinding the cells with dry ice in a mortar and pestle. The sclerotia were broken by hand grinding in a tissue grinder for 30 min to 1 h, the process of breakage being monitored with a microscope.

Lysozyme digestion. Treatment of sclerotia with lysozyme was carried out by shaking at 37 C on three successive days with a fresh 0.1 to 0.2% lysozyme solution in 0.1 M phosphate buffer (pH 7.0). The sclerotia were washed twice with 0.1 M phosphate buffer (pH 8.0) and once with water between each digestion. Hyphae were treated with lysozyme twice as described above, after which time the cell mass had diminished by about 95%.

Chromatography of amino acids, sugars, and polyols. For paper chromatography the following systems were used: for sugars and polyols, *n*-butanol-pyridine-water-toluene (5:3:3:4) (37) (system 1); for amino compounds and polyols, *n*-butanol-pyridine-water-glacial acetic acid (60:40:30:3) (system 2) or methanol-water-10 N HCl-pyridine (80:17.5:2.5:10) (system 3). Polyols were revealed using ammoniacal silver nitrate or the Bean-Porter reagent (3); other compounds were detected with ninhydrin (amino acids and amino sugars) or acid aniline phthalate (sugars) (4).

Dinitrophenyl derivatives of amino acids. Dinitrophenyl (DNP) derivatives were prepared from pure substances or from the mixture of amino acids resulting from hydrolysis of the sclerotial wall using 1 ml of 1% 1-fluoro-2,4-dinitrobenzene (FDNB) in ethanol and 10 ml of 1.6% $K_2B_4O_7$ per mg of amino acid and heating 1 h in a water bath at 60 to 65 C. The

reaction mixture was first extracted with ether to remove excess FDNB, acidified with 2 ml of concentrated HCl, and extracted with 1 ml of isopropyl acetate (IPA_c). The IPA_c extract was applied to a column of silica gel (J. T. Baker, Phillipsburg, N. J., 60-200 mesh), and the DNP from the reaction was eluted with the same solvent. The DNP amino acids were then eluted with methanol. Alternatively, purification and separation of the DNP derivatives were effected by preparative thin-layer chromatography on $PF_{2.54}$ (Brinkmann Instruments, Inc., Westbury, N. Y.) in a chloroform:tert-amyl alcohol:glacial acetic acid (70:40:3) system (system 4) and elution of the bands with methanol. The DNP derivatives were also chromatographed on Whatman no. 1 paper in a tert-amyl alcohol:pyridine:glacial acetic acid:water (110:1.0:0.08:25) mixture (system 5) (18) for 18 h at 25 C. All chromatography was carried out in the dark.

Determination of isomer of 2,3-diaminopropionic acid. The mixture of amino acids from acid hydrolysates of strain V17676 sclerotia were separated by preparative paper chromatography on water-washed Whatman no. 3 paper in system 3 by developing for 5 to 6 h at 25 C and drying the sheets overnight in a current of air. The amino acids were located by spraying a small strip from the center of the sheet with ninhydrin and eluting the appropriate bands with distilled water. The aqueous eluate containing 2,3-diaminopropionic acid (DAP_r) (admixed with some L-2,6-diaminopimelic acid) was taken to dryness in vacuo. The residue, containing approximately 300 μ g of DAP_r , was incubated with shaking at 37 C for 24 h in 1 ml of pH 8.3 $Na_2P_2O_7$ buffer (1.78 g $Na_2P_2O_7 \cdot 10H_2O$ in 180 ml water, adjusted to pH 8.3 with 1 N HCl and diluted to 200 ml) containing 0.02 ml of D-amino acid oxidase (crystalline, Sigma Chemical Co., St. Louis, Mo.) and 0.01 ml of a 1:50 dilution of catalase (type C100, Sigma Chemical Co.) and tested for disappearance of the amino acid by chromatography.

Lipids. For comparison of total lipid content, air-dried cell material which had been ground with a mortar and pestle was extracted for 1 week at 25 C with occasional shaking using a succession of solvents—methylene chloride-methanol (1:1), chloroform, and petroleum ether (boiling point 40-50 C)—and then saponified with 0.5% ethanolic potassium hydroxide for 18 h at 37 C. The various extracts were taken to dryness in a stream of nitrogen and weighed. The butter-yellow lipids extracted from the sclerotia were saponified by heating on a steam bath for 5 min with 2% methanolic potassium hydroxide. The methanol was removed in vacuo with mild heat, and a mixture of distilled water and methylene chloride (1:1) was added. After acidification of the aqueous phase with 2 N H_2SO_4 to pH 2.0, the methylene chloride-soluble material was methylated using boron trichloride-methanol, purified by preparative thin-layer chromatography, and the methyl esters of the fatty acids were analyzed by gas chromatography as previously described (28).

The various steps of this procedure were monitored by thin-layer chromatography on Silica Gel G or $PF_{2.54}$ in a petroleum ether (boiling point 40-50 C):diethyl ether:glacial acetic acid system (85:15:1) (system 6) using standard mono-, di-, and triglycerides,

fatty acids, fatty acid methyl esters, and long-chain alcohols for comparison. Polyols freed during saponification were desalted on a column of Amberlite MB-3 and identified as described in a previous section. Phospholipids were visualized using the molybdenum blue reaction of Dittmer and Lester (31) and amino acid-containing lipids with 0.4% ninhydrin in water-saturated butanol. Standards were purchased from Applied Science Laboratories (State College, Pa.) and Analabs (North Haven, Conn.). Solvomercuration-demercuration was carried out overnight at room temperature on the fatty acid methyl esters by the procedure described by Gerber (14), modified by substituting 1% NaHCO_3 for the 10% NaOH to prevent saponification of the esters.

Heat resistance. Vegetative and sclerotic cells were obtained as described above for cell-wall analysis. The cells were washed twice in sterile 0.1 M phosphate buffer (pH 7.0), resuspended in buffer, and heated in a water bath at 60 C for 4 h, 70 C for 15 and 30 min, and 96 C for 1 min. At the end of this period the tubes were cooled immediately, inoculated onto YD agar, and incubated 3 weeks at 28 C.

Cells of *Streptomyces fradiae* IMRU 3535 grown shaken in YD liquid culture for 24 h were included for comparison.

Electron microscopy. Sclerotia were fixed on ice in either the formaldehyde-glutaraldehyde mixture of Karnovsky (23) for 1 to 18 h or the glutaraldehyde-osmium tetroxide mixture of Franke et al. (11); both were buffered with 0.1 M sodium cacodylate. After several buffer rinses, the sclerotia were placed in 1% buffered osmium tetroxide for 2 to 3 h on ice rinsed several times with buffer and then with distilled water. After 3 h at room temperature in 0.5% aqueous uranyl acetate, they were rinsed in distilled water and dehydrated either by (i) passages through increasing concentrations of ethanol followed by absolute acetone or (ii) increasing concentrations of hexylene glycol. The material was gradually infiltrated with Araldite 6005 by the method of Richardson et al. (33). Alternatively, the low viscosity epoxy resin described by Spurr (36) was used. Polymerization was carried out at 60 to 70 C for 20 to 60 h. Sections were stained for 10 to 20 min with lead citrate (32) and examined with a JEM 7A/120 electron microscope operated at 80 kV.

Observation of spore surface. Formvar-coated grids were dropped on part of *Chainia* colonies which had aerial hyphae. Some chains of conidia stuck to the Formvar and were observed with an Akashi TRS-50 electron microscope.

Thick sectioning. Araldite-embedded colonies were glued to a plastic rod which was held with a jeweler's vise and placed in the field of vision of a 40X stereoscopic microscope. Sectioning was done free-hand with a fine scalpel. Sections were mounted under cover slip in immersion oil and were observed with a phase-contrast microscope.

RESULTS

Chainia sp. strain V17676 was sent to us by E. Tejera because of the beauty of the sclerotia formed and the abundance of lipid they contained. Comparison of the physiology and

morphology of strain V17676 with other strains of *chainiae* showed it to be closest to two strains of *C. olivacea* (S and 3751) (Table 1). *Chainiae* are similar to streptomycetes in cell wall composition and in mode of sporulation; however, none of the strains tested could be identified with any of the species of *Streptomyces* which have been characterized by the methods of Gordon (16).

C. olivacea V17676 slowly lost its ability to form sclerotia upon continued cultivation on laboratory media. During the course of this study, fresh strains were obtained periodically from our freeze-dried stock. When formed, sclerotia were abundantly produced on PC, Cz, and YD agars. Production of sclerotia was best on TPab and TPC agars where hyphal growth was minimal. In old plate cultures (2-week-old or more), the surface of the agar was covered with a thick layer of yellow sclerotia which could be easily scraped off.

When spores and hyphal fragments were placed on TPab agar, hyphal development took place with the formation of radiating colonies typical of actinomycetes. After 18 to 20 h it was difficult, using a light microscope, to resolve the individual hyphae at the center of the colonies (Fig. 1a) at the location where, upon further incubation (Fig. 1b), the sclerotia would eventually grow. These sclerotia were at first tan and then, on maturing, yellow. Normally a number of sclerotia were formed at the center of a colony (Fig. 1c). As the colony grew, the sclerotia increased in size and new sclerotia initials were formed at some distance from the center maturing into a "fairy ring" of sclerotia (Fig. 1d). In some large and old colonies, more than one such fairy ring could be produced. Four-day-old sclerotia were spherical and 50 to 75 μm in diameter.

If a sclerotium was removed with a sterile fine point and placed on the surface of a TPab agar plate, germination of the sclerotium did not take place; rather, the few hyphae present on the surface of the sclerotium grew out radially and, after a few hours, the colony so formed was similar to the one from which the sclerotium had come. In other terms, a sclerotium on a nutritive medium could produce a colony without apparent use of its own nutritional resources, and the colony formed from a sclerotium could not be distinguished from one which had formed such a structure. Sclerotia placed on WA prepared with Noble agar and distilled water behaved in the same fashion, indicating that there were still enough impurities in the medium to allow growth of the actinomycetes without apparent wasting of the sclerotia.

After 9 days of incubation on TPab, numer-

FIG. 1. Steps in the formation of sclerotia by *Chainia olivacea* strain V17676 on solid media. a, Young colony, 15-h-old. Hyphal structure visible in the whole colony except the very center. b, 20-h-old. Sclerotia initials are developing on top. c, 24- to 48-h-old. Sclerotia well developed. Coils of aerial hyphae are visible. d, 48 h and more. Formation of a fairy ring of new sclerotia.

ous tufts of white aerial hyphae were observed which contained chains of conidia in open spirals. Observed with an electron microscope, the conidia were found to have a smooth surface.

When sclerotia of *C. olivacea* were crushed between a slide and a cover slip, many oil droplets were released. If the pressing was thorough enough to dislodge most of the oil, a honeycomb-shaped plurilocular structure was observed (Fig. 2).

When grown in Cz shaken liquid medium at 28 C, the organism formed sclerotia-like bodies which were usually more elongated than spherical (about 100 by 250 μ m). Upon crushing, these pseudosclerotia released oil droplets and a mass of short hyphae. No plurilocular structure was observed.

To gain some insight into the mode of formation of sclerotia, 20-h-old colonies grown on TPab agar at 25 C were excised, fixed, dehydrated, and embedded in Araldite resin. At that age, the colonies were just starting to show signs of differentiation at their centers as in Fig. 1b. These resin-embedded colonies were first sectioned free hand, and the sections were observed by phase microscopy. The alveolar structure of the sclerotia initials could be seen surrounded by a granular area where the hyphal structure could no longer be resolved (Fig. 3); further away, the hyphae were clearly visible.

Ultrathin sections were made and observed with the electron microscope. The first step in the formation of sclerotia is the appearance of numerous amorphous inclusions in hyphae (Fig. 4). These are probably oil droplets. The lipid-rich hyphae contain numerous mesosomes and undergo repeated divisions leading to a cellular-like structure (Fig. 5, 6, and 7), which may be equated with the granular region seen in light microscopy surrounding the plurilocular area. As the cells fill with lipid, an amorphous material is deposited between the cell walls (Fig. 8). This amorphous material probably becomes the matrix of the plurilocular structure (PS). As the sclerotium matures further, the walls of the PS become increasingly more difficult to section. In a given sclerotium, the center, presumably the most mature portion, was the hardest to section. The cells of the PS became completely filled with lipids (Fig. 9), and eventually regrowth of hyphae took place in the cells (Fig. 10). (The overall process is summarized schematically in Fig. 11 a to e.)

All the *Chainia* strains listed in Table 1 had a Type I cell wall (26). In the case of V17676, it was found, however, that hydrolysates of the

walls of sclerotia as well as of hyphae contained an additional amino acid besides those characteristic of cell walls of Type I. This compound has been shown to be L-2,3-diaminopropionic acid (L-DAP_r). The free amino acid was compared with authentic DAP_r on the basis of color reaction with ninhydrin (bright blue when heated at about 120 C) and rate of migration in chromatography systems 2 and 3. In system 2, both compounds had an $R_{\text{glutamic acid}}$ of 0.84 after 48 h. In system 3 they had an $R_{\text{L-diaminopimelic acid}}$ of 0.93. The mixture of DNP derivatives of the cell-wall amino acids showed a spot migrating with authentic DAP_r-DNP in systems 4 and 5. Incubation of purified DAP_r isolated from hydrolysates of sclerotia with D-amino acid oxidase showed no disappearance of the compound; hence, its isomeric configuration is of the L series.

Other strains of *Chainia* species, namely *C. antibiotica* and *C. purpuregena*, from which sclerotia could be obtained in large amounts, also contained DAP_r. It is also of interest that hyphae from submerged growth of a V17676 substrain which had largely lost its capacity to form sclerotia showed only traces of this compound, whereas hyphae of a substrain still producing sclerotia contained substantial amounts. It is possible that the DAP_r is contained in the "cement" between the cells.

The amount of lipids contained in the sclerotia and hyphae of V17676 is presented in Table 2. Similar figures for lipids extracted from dried cells of representatives of the genera *Mycobacterium* and *Nocardia*, both of the order *Actinomycetales* and both known to produce large amounts of these substances, are included for comparison. As can be seen, V17676 is extraordinarily lipid rich. The *Chainia* figures are from cells grown on Czapek's medium (agar for sclerotia, liquid for hyphae), a medium in which sclerotia production (and hence lipid production) is optimal. The *Mycobacterium* and *Nocardia* strains were grown on YD because they do not grow well on Czapek's liquid. The lipid content of the *Mycobacterium* would probably increase on longer incubation. An average of 19% (dry weight) for eight strains of mycobacteria grown in Sauton's medium for 4 to 6 weeks has been reported (1). Members of the genus *Streptomyces*, which is closely related to *Chainia*, contained only 0.56 to 2.7% lipids (1).

The lipids of *Chainia olivacea* strain V17676 are triglycerides of branched-chain fatty acids of the iso/anteiso series, principally C₁₅ and C₁₆ with lesser amounts of C₁₃, C₁₄ and C₁₇.

FIG. 2. Crushed 48-h-old sclerotium of *C. olivacea* grown on TPab. Immersion objective, phase contrast. Note plurilocular structure and oil droplets. Bar, 10 μ m.

FIG. 3. Section through an 18-h-old sclerotium of *C. olivacea* grown on TPab. Immersion objective, phase contrast. Note plurilocular structure with adjoining granular area. Bar, 10 μ m.

FIG. 4. Branched hypha found in sclerotium-forming cultures of *C. olivacea* grown on TPab for 6 days at 28 C. Large inclusions which appear to be lipid droplets nearly fill the cell. Fixation was for 18 h in a glutaraldehyde-osmium tetroxide solution, followed by 3 h in OsO_4 and 3 h in uranyl acetate. Dehydration was in ethanol, then acetone; embedding was in Araldite 6005. Sections were stained with lead citrate. Bar, 1 μ m.

FIG. 5. Thin section through a young sclerotium of *C. olivacea* grown on TPab for 20 h at 28 C. Sclerotia were fixed in a formaldehyde-glutaraldehyde mixture for 17 h, followed by 2 h in OsO_4 , and 3 h in uranyl acetate. Dehydration was in hexylene glycol, and embedment was in the "A" mixture of Spurr. Sections were stained with lead citrate. Bar, 1 μ m.

FIG. 6. Thin section through a young sclerotium of *C. olivacea*. Fixation and staining as for specimen shown in Fig. 5. Bar, 1 μ m.

FIG. 4 to 6.

FIG. 7. Thin section through a scleritum of *C. olivacea* grown on TPab for 7 days at 28 C. Fixation was in formaldehyde plus glutaraldehyde for 18 h followed by OsO_4 for 2 h, and then 3 h in uranyl acetate. Dehydration was in hexylene glycol. Spurr "E" mixture was used for embedding. Staining of the sections was with lead citrate followed by 5 min in 2% aqueous barium permanganate. Bar, 1 μm .

FIG. 8. Thin section of a scleritum of *C. ochracea* grown on TPab at 28 C for 6 days and then fixed in formaldehyde and glutaraldehyde for 18 h. Postfixation was in OsO_4 for 3 h followed by staining in uranyl

FIG. 11. Steps in the formation of sclerotia of *C. olivacea*: young hypha (a); hypha becoming filled with lipid inclusions (b); hyphae swell and divide, forming a mass of lipid-rich cells, mesosomes are numerous (c); deposition of intercellular cement (d); all cellular material has been transformed into lipids, there is regrowth of a few hyphae in some of the cells (e).

acetate for 3 h. Dehydration was in ethanol followed by acetone, and embedment was in Araldite 6005. Sections were stained with lead citrate. Bar, 1 μ m.

FIG. 9. Thin section of a sclerotium of *C. ochracea* grown on YD for 6 days at 28 C. The preparative procedure was like that described for the specimen shown in Fig. 8 with the exception of the initial fixative, which was glutaraldehyde and osmium tetroxide in this instance. Bar, 1 μ m.

FIG. 10. Thin section of a sclerotium of *C. ochracea* grown on TPab for 7 days at 28 C. Fixation was in formaldehyde and glutaraldehyde for 18 h followed by OsO_4 for 2 h and then staining with uranyl acetate at room temperature for 3 h. Dehydration was in ethanol and acetone, and embedment was in Spurr mixture "E." Sections were stained with lead citrate and barium permanganate. Bar, 1 μ m.

TABLE 1. *Biochemical properties of strains of Chainia*

Strain ^a	Hydrolysis of xanthine	Utilization of oxalate	Production of:		Acid from:										Growth at:		Survival: 50 C, 8 h
			Urease	Nitrate reductase	Adonitol	Erythritol	Inositol	Lactose	Mannitol	Melibiose	α -Methylglucoside	Raffinose	Rhamnose	Xylose	10 C	42 C	
<i>C. antibiotica</i> Su 7 (S) ^b	+	+	+	—	+	+	+	+	+	+	+	+	+	+	—	+	+
<i>C. antibiotica</i> 3750	+	+	+	+	—	—	+	+	+	+	+	+	+	+	—	+	+
<i>C. aurea</i> Su 8 (S)	—	—	+	—	—	—	—	+	+	—	+	—	+	+	—	+	+
<i>C. flava</i> Su 9 (S)	—	+	+	—	—	—	—	+	+	—	+	—	+	+	—	+	+
<i>C. fumigata</i> SU 10 (S)	+	+	+	—	—	—	—	+	+	+	+	+	+	+	—	+	+
<i>C. minutisclerotica</i> Su 11 (S)	—	+	+	—	—	—	+	+	+	—	+	—	+	+	—	+	+
<i>C. nigra</i> Su 12 (S)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+
<i>C. ochracea</i> Su 13 (S)	+	+	+	—	+	+	+	+	+	+	+	—	+	+	+	—	+
<i>C. olivacea</i> Su 14 (S)	+	+	—	—	—	+	+	+	+	—	+	+	+	+	—	—	+
<i>C. olivacea</i> 3751 Lyo ^c	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+
<i>C. olivacea</i> 3751	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+
<i>C. poonensis</i> Su 15 (S)	+	+	—	+	—	—	+	+	+	—	+	—	+	+	—	—	+
<i>C. poonensis</i> 3752	+	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—
<i>C. purpureogena</i> Su 16 (S)	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. rosea</i> Su 17 (S)	—	+	—	—	—	—	+	+	+	+	+	—	+	+	—	+	+
<i>C. olivacea</i> V17676	+	+	+	+	+	+	+	+	+	—	+	+	+	+	—	—	+
<i>C. olivacea</i> V17676 Lyo	+	+	+	+	+	+	+	+	+	—	+	+	+	+	—	—	+

^a For all strains, hydrolysis of casein, hypoxanthine, adenine, starch and gelatin was positive; utilization of citrate, malate and succinate was positive; none of the strains grew in salicylate broth; acid was produced from galactose, glucose and mannose; all strains grew at 37 C; none grew at 53 C.

^b S, From R. S. Sukapure.

^c Culture freshly revived from freeze-dried state.

TABLE 2. Total lipids of *Chainia olivacea* V17676 and other actinomycetes

Strain	Determination			
	Dry wt of cells extracted (g)	Methanol-methylene chloride extract (g) ^a	Chloroform extract (g)	Methylene chloride solubles after saponification (g)
<i>C. olivacea</i> V17676 Sclerotia Hyphae ^b Hyphae ^c	1.5200	0.6280	0.0224	0.1000
	0.7320	0.2320	0.0130	0.0283
	0.9332	0.2670	0.0156	0.0296
<i>Nocardia asteroides</i> IMRU 727 hyphae	1.0007	0.0206	0.0075	0.1047
<i>Mycobacterium smegmatis</i> IMRU 375 hyphae	1.0000	0.0328	0.0373	0.0716
				50 37 33 13 14

^a Portion of residue soluble in methylene chloride. ^b Hyphae of sclerotia-forming strain grown in submerged culture. ^c Hyphae of non-sclerotia-forming strain.

This pattern is similar to the type of fatty acids reported from the closely related streptomycetes (1). The identification of the fatty acids was based on retention times of the methyl esters compared with authentic compounds on GLC and the lack of reaction in the solvomercuration-demercuration reaction. Glycerol was identified by its R_F in systems 1 and 2 by comparison with the known compound and by its reaction with the Bean-Porter reagent.

On thin-layer chromatography (TLC) in system 6, the crude lipid extracted from V17676 sclerotia migrated as a single major spot corresponding in R_F to standard triglycerides. No compounds reacting with ninhydrin or the molybdenum blue reagent for phospholipids were found. Saponification of the crude lipid yielded compounds migrating on TLC plates like normal fatty acids. Methylation of the saponified residue yielded a mixture which showed one major spot on TLC corresponding to the methyl esters of normal fatty acids and two minor, more polar, spots of unknown composition, possibly corresponding to the α -hydroxy fatty acids reported from streptomycetes (25).

Some strains of *Chainia* have been isolated from relatively hostile environments such as deserts (personal communication, R. S. Sukapure), but this is not true for all strains of *Chainia*, including V17676. In an attempt to determine whether the sclerotia play a role in heat resistance, both sclerotia and vegetative cells of V17676 were compared with the vegetative cells of *Streptomyces fradiae* IMRU 3535. The results (Table 3) show that sclerotia are not only more heat resistant than cells of the streptomycete but also more resistant than hyphae of V17676 itself.

Prolonged digestion of sclerotia with lysozyme did not alter their appearance when viewed with the light microscope. However, acid hydrolysates of such sclerotia which had been delipidated by extraction with methylene chloride showed that the muramic acid, glucosamine, and L-diaminopimelic acid had largely disappeared, as did some of the glutamic acid. The major components remaining were DAP_r, aspartic acid, lysine, serine, glycine, alanine, and galactosamine, which we assume are the main constituents of the intercellular "cement."

DISCUSSION

2,3-Diaminopropionic acid (β -amino alanine) has been found previously in nature but not, as

TABLE 3. Heat resistance of sclerotia and hyphae of *Chainia olivacea* V17676 and of hyphae of *Streptomyces fradiae* IMRU 3535

Strain	60 C/4 h	70 C/15 min	70 C/30 min	96 C/1 min
V17676				
Sclerotia	+	+	+	—
Hyphae	—	—	—	—
3535				
Hyphae	—	—	—	—

^a +, Growth.^b —, No growth.

far as we know, in the structural components of a microorganism. There have been reports of this amino acid either free or oxalylated from seeds of plants (6, 15, 30, 42). It has also been found free (as the D isomer) in the digestive fluid of *Bombyx mori* (43) and as a constituent of the antibiotics edeine (L-isomer) (34), viomycin (L-isomer) (19, 43), and capreomycin (20). The last two antibiotics are produced by actinomycetes.

Structures formed by eubacteria which are resistant to adverse conditions include endospores and cysts. Actinomycetes are bacteria having a fungoid morphology; thus, they form not only endospores (9) like bacteria but also sclerotia, like fungi. The structure of the sclerotia of *Chainia* is different from that of other structures formed by actinomycetes. Perhaps it is closest to that of the vesicles of the endophytes now placed in the genus *Frankia* (5, 12).

In fungi, sclerotia are firm aggregations of vegetative hyphae containing reserve materials. They can be rich in carbohydrates, such as those of *Phymatotrichum omnivorum*, or in lipids, such as those of *Claviceps purpurea*. After maturity, sclerotia are capable of independent persistence. They germinate at the expense of the reserve materials by producing either hyphae, as in *P. omnivorum*; spores, as in species of *Botrytis*; or carpophores, as in species of *Claviceps* (7, 8).

The heat tolerance of sclerotia of strain V17676 supports the assumption that formation of sclerotia by chainiae helps these organisms to survive under adverse conditions. The heat resistance of the sclerotia is, however, quite modest when compared with that of spores of *Thermoactinomyces vulgaris*, which can withstand 45 min at 100 C (9). It is essentially of the same order of magnitude as that of spores of streptomycetes (45). We have not been able to demonstrate that the lipids

stored in the sclerotia of strain V17676 were actually used for growth. Even on the most meager medium we used, some growth took place without any apparent wasting of the sclerotia. This may be due to the fact that actinomycetes in general are able to grow at the expense of only minute amounts of nutrients or that the rigid structure of sclerotia does not break down even though the lipids are utilized.

In light of our results, the actual role of sclerotia of strains of *Chainia* is not clear. The sclerotia are somewhat heat resistant and contain substantial amounts of triglycerides; however, the role of the lipids as storage materials is not obvious. The production of sclerotia probably does not confer any apparent ecological advantage over the production of spores of the type produced by streptomycetes.

Should sclerotia-forming organisms with a cell wall of Type I (*Streptomyces* type) be placed in a special genus? The property of producing sclerotia is easily lost upon cultivation on laboratory media, and a nonsclerotial strain of *Chainia* cannot be differentiated from a streptomycete. In addition, to complicate matters further, some members of the order *Actinomycetales* with Type IV cell walls also produce sclerotia (M. P. Lechevalier, unpublished results). However, until it has been demonstrated experimentally that "ordinary streptomycetes" can be induced to form truly plurilocular sclerotia, we favor recognizing *Chainia* as a distinct genus.

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