A death round affecting a young compartmentalized mycelium precedes aerial mycelium dismantling in confluent surface cultures of *Streptomyces antibioticus*

Ángel Manteca,¹ Marisol Fernández² and Jesús Sánchez¹

Correspondence

Jesús Sánchez

jsm@fq.uniovi.es

¹Area de Microbiología, Departamento de Biología Funcional, Facultad de Medicina, Universidad de Oviedo, Julián Clavería s/n, 33006 Oviedo, Spain

²Laboratorio de Proteómica, Centro Nacional de Biotecnología, Cantoblanco, 28049 Madrid, Spain

Received 17 March 2005

Revised 20 June 2005

Accepted 26 July 2005

Development-associated cell-death processes were investigated in detail during the growth and differentiation of *Streptomyces antibioticus* ATCC 11891 on confluent surface cultures, by using fluorescent viability probes, membrane and activity fluorescence indicators, and electron microscopy analysis. A previously unsuspected complexity was revealed, namely the presence of a very young compartmentalized mycelium that dies following an orderly pattern, leaving alternating live and dead segments in the same hypha. This death round is followed by the growth of a second mycelium which develops rapidly from the live segments of the first mycelium and dies massively in a second death round, which extends over the phases of aerial mycelium formation and sporulation.

INTRODUCTION

Streptomyces forms two differentiated structures when grown as surface cultures (Waksman, 1967): a substrate (vegetative) mycelium and an aerial (reproductive) mycelium. The substrate mycelium is usually described as a branched structure with hyphae that have a mean diameter of 0.7 µm, bounded by a mucopeptide cell wall 0.01–0.02 µm wide and crossed with relatively scarce septa which delimit compartments usually containing many nucleoids (Waksman, 1967; Hodgson, 1992; Chater, 1993; Chater & Losick, 1997). This mycelium is assumed to be present, though in different stages of cellular degeneration, during all growth phases, which in *Streptomyces antibioticus* cultures last for about 90 h of cultivation on solid medium (Mendez *et al.*, 1985; Braña *et al.*, 1986; Miguelez *et al.*, 1999). After about 35 h or more of cultivation, a mycelium network develops into the air that grows and produces chains of spores with hydrophobic surfaces (Chater, 1984). In early studies, aerial hyphae were reported as originating from simple branching from substrate hyphae (Hopwood & Glauert, 1961), and they are preceded by a short period of reduced macromolecular synthesis (Granozzi *et al.*, 1990).

Bacterial death phenomena in communities appear to be active processes related to a multicellularity trait likewise subject to environmental factors and developmental processes (Rice & Bayles, 2003). Lytic phenomena associated with development were reported early on in *Streptomyces coelicolor* A3(2) by Wildermuth (1970), who described two central developmental fates for the *Streptomyces* mycelium: the surface layer, leading to spore formation, and the underlying, non-sporulating hyphae, leading to lysis. This phenomenon was subsequently interpreted by assuming that the lysis of the substrate mycelium could serve the purpose of providing nutrients for the developing aerial structures (Chater, 1984; revised by Hodgson, 1992). Analysis of the mobilization of radioactively labelled amino acids during the development of *S. antibioticus* ATCC 11891 on surface cultures supports the reutilization hypothesis (Braña *et al.*, 1986). The existence of an orderly process of internal cell dismantling was further addressed in *S. antibioticus* ATCC 11891 by electron microscopy analysis of the mycelium growing in confluent lawns (Miguelez *et al.*, 1999).

In this paper, we present a complete morphological analysis of the main developmental stages that accompany the growth of confluent surface cultures of *S. antibioticus* ATCC 11891. To obtain a reliable picture of the cell-death phenomenon, which constitutes the *Streptomyces* developmental hallmark, we used previously assayed viability staining with propidium iodide (PI) and SYTO 9 (Fernandez & Sanchez,

Abbreviations: CFDA, carboxyfluorescein diacetate; CLSM, confocal laser-scanning fluorescence microscopy; FM 4-64, *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenyl)-hexatrienyl pyridinium dibromide; PI, propidium iodide; WGA, wheat germ agglutinin.

2001, 2002). This technique has been widely used for determining membrane integrity in bacteria (Miller & Quarles, 1990; Lloyd & Hayes, 1995; Bunthof *et al.*, 1999, 2001) and involves staining the nucleic acids of the damaged (leaky) cells with PI. The study was complemented by other fluorescence and electron microscopy analyses. A number of interesting features were uncovered by our work, such as the existence of very early cell death in a young compartmentalized mycelium. The availability of a reliable model for the development-associated death processes may now enable the genetic analysis of this interesting phenomenon, which is central to the biology of the bacterium.

METHODS

Strains and media. *Streptomyces antibioticus* ATCC 1181 was the species used in this study. The micro-organism was grown in solid glucose/asparagine/yeast extract (GAE) medium (Braña *et al.*, 1986), in which it presents a complete life cycle with abundant sporulation. The cultures were prepared in Petri plates (8.5 cm diameter) as lawns on solid GAE medium (30 ml per plate) at 30 °C. Plates were directly inoculated with 100 µl of a spore suspension (1.5×10^7 viable spores ml⁻¹), followed by incubation at 30 °C.

Obtaining protoplasts from surface cultures. Protoplasts were obtained from the *S. antibioticus* compartmentalized mycelium as described elsewhere for submerged cell cultures (Okanishi *et al.*, 1974; Kieser *et al.*, 2000). Young mycelium grown on a cellophane disc was scraped off, resuspended in 10.3% sucrose solution (about 0.6 g sucrose per 15 ml), and washed twice by centrifugation at 10 000 r.p.m. The pellet was resuspended in 4 ml buffer P plus lysozyme (2 mg ml⁻¹, Sigma) and incubated at 37 °C for about 30 min. The formation of protoplasts was monitored under a phase-contrast microscope. When appropriate, the protoplasts were visualized after staining under the confocal laser-scanning fluorescence microscope, as described below.

Viability staining. Culture samples were obtained and processed for microscopy at different times of incubation, as described previously (Fernandez & Sanchez, 2001, 2002). GAE plates were prepared with Difco agar, inoculated as described above and used to obtain solid blocks of the agar cultures with a scalpel. These were further trimmed to squares of about 10 mm in size and introduced into a hand microtome (11 mm hole diameter) previously cooled to 4 °C, with the growth surface oriented towards the side. Sections of about 0.3 mm were obtained. The limit of the agar and the limit of *Streptomyces* mycelium were defined at the lateral edge of the sample by fluorescence and/or phase-contrast observation. To improve visualization of the individual hyphae in some of the developmental phases, the stained samples were squashed in the slide by applying a gentle pressure on the coverslip. The mycelium becomes disorganized and loose, allowing the observation of specific details of the hyphae. The permeability assay described previously for *Streptomyces* was used to stain samples (Fernandez & Sanchez, 2002). This involves staining the cells with a cell-impermeant nucleic acid stain (PI) in order to detect the dead cell population of *S. antibioticus*, and with SYTO 9 green fluorescent nucleic acid stain (LIVE/DEAD BacLight Bacterial Viability Kit, Molecular Probes, L-13152) to detect the viable cells. The SYTO 9 green fluorescent stain labels all the cells, i.e. both those with intact and those with damaged membranes. In contrast, PI only penetrates bacteria with altered membrane permeability and presents substantial fluorescence enhancement upon binding nucleic acids. This causes a reduction in the SYTO 9 stain fluorescence when both dyes are present. Thus, in the presence of both stains, bacteria with intact cell membranes appear fluorescent

green, whereas membrane-compromised bacteria appear red (Haugland, 2002). The stain mix was prepared as recommended by the manufacturer and was added directly over the samples on the slide. For the viability in liquid cultures assay, a drop of the above mixture was added to a drop of the liquid mycelial sample (Fernandez & Sanchez, 2001). The coverslip was placed over the surface and submerged samples, staining taking place for at least 10 min in the dark. The samples were observed under a Leica TCS-SP2-AOBS confocal laser-scanning microscope at wavelengths of 488 nm and 568 nm excitation and 530 nm (green) or 630 nm (red) emission. Images were mixed using the Leica Confocal Software. In some cases, the samples were also observed in differential interference contrast mode, available with the same equipment.

Membrane staining. Lipophilic styryl dye, *N*-(3-triethylammonium-propyl)-4-(*p*-diethylaminophenyl-hexatrienyl) pyridinium dibromide (FM 4-64) (Molecular Probes, T-3166), was added directly to the culture medium before pouring into the plates (Vida & Emr, 1995) at a final concentration of 1 µg ml⁻¹ that did not affect growth. At the appropriate times, culture samples were obtained and processed for microscopy, as described above for viability staining, and observed under the confocal laser-scanning microscope at wavelengths of 550 nm excitation and 700 nm emission.

Activity cell staining. *S. antibioticus* was grown on the surface of cellophane discs and scraped off with a plain spatula at different times. The harvested mycelium was incubated at 30 °C in a carboxy-fluorescein diacetate (CFDA) solution (Molecular Probes, C-1157) at a final concentration of 30 µM for 15 min, washed twice with distilled water and observed directly under the confocal laser-scanning microscope at wavelengths of 488 nm excitation and 530 nm emission. The samples were sometimes additionally stained with PI, after CFDA staining.

Analysis of cell membrane potential with rhodamine-123. Rhodamine-123 is a vital stain that selectively accumulates in cells with an intact membrane potential (Haugland, 2002) and has been widely employed in both eukaryotic (Darzynkiewicz *et al.*, 1992) and prokaryotic (Lopez-Amoros *et al.*, 1995) cell studies. Mycelium harvested from cellophane surface cultures of *S. antibioticus* was incubated at 30 °C for 15 min in a solution of rhodamine-123 (0.2 mg ml⁻¹ final concentration, Sigma), washed twice with distilled water and observed under the confocal microscope at wavelengths of 514 nm excitation and 563 nm emission. Some samples were additionally tested for viability with PI and SYTO 9 staining. In this case, the rhodamine emission was recorded at 580 nm in order to prevent interference with the SYTO emission spectrum.

Cell wall staining. Wheat germ agglutinin (WGA) conjugated with Texas red (Molecular Probes W-21405), which binds selectively to *N*-acetylglucosamine and *N*-acetylneuraminic acid, was extensively used to stain cell walls. Solid cultures of *Streptomyces* were scraped from cellophane with a plain spatula at various times. Harvested mycelium was processed as described elsewhere (Schwedock *et al.*, 1997). Cells were fixed in 2.8% paraformaldehyde, 0.0045% glutaraldehyde in PBS (0.14 M NaCl, 2.6 mM KCl, 1.8 mM KH₂PO₄ and 10 mM Na₂HPO₄) for 15 min at room temperature, washed twice with PBS and incubated for 1 min in 2 mg lysozyme ml⁻¹ in glucose/Tris/EDTA (GTE: 50 mM glucose, 20 mM Tris/HCl, pH 8, 10 mM EDTA). The samples were washed again with PBS and blocked in 2% BSA in PBS for 5 min. WGA was added at a concentration of 100 µg ml⁻¹ in 2% BSA in PBS, and incubated at room temperature for 3 h. Finally, the samples were washed eight times with PBS and observed under the confocal microscope at wavelengths of 595 nm excitation and 615 nm emission.

Electron microscopy. Samples of the cultures grown on the surface of cellophane discs were scraped off at different times of

incubation with a plain spatula and incorporated in 3 % agar blocks. These were cut into small pieces and fixed at 4 °C in 2.5 % glutaraldehyde. After washing in 0.1 M phosphate buffer, pH 7.3, the cells were postfixed overnight at 4 °C in a solution of 1 % (w/v) osmium tetroxide in 50 mM phosphate buffer pH 7.3. Pieces were dehydrated through graded acetone solutions over a 2 h period at room temperature. In the 70 % dehydration step, 2 % (w/v) uranyl acetate was added and the samples were left for 1 h to contrast the cells. After completion of the dehydration treatment, the blocks were embedded in London Resin White (LR-White) and polymerized at 60 °C for 40 h. Ultrathin sections of silver-grey interference colour (thickness 60–90 nm) were obtained for electron microscopy observations using a diamond knife and mounted on Formvar-coated copper grids. Samples were examined under a Philips EM300 electron microscope at an operating voltage of 60 kV and photographed with Scientia electron microscopy film (AGFA).

RESULTS

Confocal laser-scanning fluorescence microscopy (CLSM) analysis of the development-associated cell-death processes of *S. antibioticus* ATCC 11891 in confluent surface cultures

A precise view of the *S. antibioticus* life cycle on surface GAE medium was obtained after analysing more than a thousand images by CLSM. The successive developmental stages are summarized in Fig. 1. Fig. 1(a, b) represents the germination and early development of the hyphae (5–10 h). The viable, green-stained spores that spread over the surface of the plates remain in more or less large groups, probably due partly to the hydrophobicity of the spores. Germination starts at around 5 h by means of the asynchronous outgrowth of a single (or less frequently, double) germ tube. Most of the young hyphae formed suffer an early death process (Fig. 1e), which generally occurs in a remarkably symmetrical form: live and dead segments alternate very regularly in the same hyphae, giving them an appearance which we refer to in this study as 'variegated'. A small proportion of spores emit a germ tube that remains totally viable during the initial hours (arrow in Fig. 1c, and Fig. 1d) but which also ends up dying in the aforementioned variegated way. The boundaries separating the compartments of this first mycelium are also visible in these live hyphae (arrows in Fig. 1d).

Fig. 1(f, g), presents a general view of the extension of the first death round (10–18 h), which at the end of the process uniformly affects this initial substrate mycelium. The hyphae within the mycelial mass suffering the death process have entered into contact with one another, forming a thin layer that grows on top of and into the agar (Fig. 1f, g). Some spores have still not germinated (Fig. 1f). Fig. 1(h–j) shows the phases in which this second mycelium develops and spreads rapidly and homogeneously along the transverse section. The red fluorescent primary mycelium has mostly disappeared at 20–22 h (Fig. 1i, j) due to the disintegration of the hyphae and nucleic acid degradation (see below).

Fig. 1(k) shows how the second mycelium grows massively until reaching the phase in which aerial mycelium is visible on the plate surface, i.e. at about 25 h. This coincides with a marked increase in the thickness of the mycelium layer [compare the scales of Fig. 1(i) with those of Fig. 1(k)]. Simultaneously, a second massive death round affecting this second mycelium takes place in the deepest zone of the section (Fig. 1k). The growth of the remaining live mycelium continues and the hyphae start to segment their DNA in nucleoids (Fig. 1l). The death waves of the mycelium gradually reach the upper zones of the section and, as a consequence, an important proportion of the DNA-segmented hyphae also appear dead at the culmination of this phase (Fig. 1l). At these times (36 h), spores are not yet being formed.

Finally, the sporulation phase takes place at 48–96 h. The spores form a thin layer on the surface of the medium and the mycelium layer presents its maximum thickness. However, as occurred in the first death round (Fig. 1i, j), no fluorescence is observed below the surface spore layer, conceivably due to the disintegration of the dead hyphae and the degradation of nucleic acids (Fig. 1m, see below). The form of the hyphae cannot be observed under the phase-contrast microscope (Fig. 1n), only a thick cellular mass that is opaque to light.

Details of the most relevant events occurring during the first 15 h are shown in Fig. 2. Fig. 2(a) represents a young compartmentalized hypha with dead and live regions marked by a defined boundary. The majority of the live segments of the variegated mycelium show a drop in the intensity of their green fluorescence (Fig. 2a, b), which is recovered in a later phase (Fig. 2d–f; see below). Fig. 2(b, c, d and f) shows details of the completion of the first death round (10–18 h), which at the end of the process uniformly affects this initial substrate mycelium. As shown also in Fig. 1(f), some spores have still not germinated in this time (Fig. 2b, c). The mycelium appears fragmented due to the disintegration of the dead segments, which can be seen covered by a diffuse red layer likely formed by the intracellular material (Fig. 2e). Some of the live segments of the variegated mycelium start to enlarge asynchronously (Fig. 2f; visible also in Fig. 1g) to form what we call a second mycelium, in order to distinguish it from the aforementioned first mycelium.

The variegated appearance of the young hyphae is caused by their compartmentalized nature

Direct evidence of the presence of septa delimiting the alternating dead and live segments in the first mycelium was obtained by using several fluorescent indicators and by electron microscopy analysis. FM 4-64 labels the plasma membrane of eukaryotic cells (Vida & Emr, 1995) and bacteria (Sun & Margolin, 1998; Pogliano *et al.*, 1999), including *Streptomyces* (Grantcharova *et al.*, 2003). Fig. 3(a) shows the mycelium stained with the membrane-specific fluorochrome FM 4-64. Stained structures transverse to the

Fig. 1. CLSM analysis of transverse sections obtained from *Streptomyces antibioticus* plate cultures at different times and developmental stages. Samples were stained with SYTO 9 and PI (see Methods). Numbers indicate growth times. Pictures (a) to (e) represent the germination phase and early development of the hyphae (see text). Spores and young hyphae can be seen in the same preparation due to asynchronous germination (a–c). The early death process of these young hyphae shows a remarkable pattern, with live and dead segments alternating regularly in the same hypha (a, e). Spores with a germ tube that remains totally viable can be seen against the background of variegated hyphae (c, arrow). These viable hyphae are also compartmentalized (arrows in d). (f) A general view of the completion of the first death round, which uniformly affects the young first mycelium. (g) Growth of the young second mycelium (arrow). (h–j) Development of the second mycelium; dead, red-fluorescent primary mycelium can be seen between the live hyphae in (h), although it is no longer visible in (i) and (j). (k) Second death round affecting the deepest zone of the mycelium layer; growth of aerial mycelium on the upper zone (green) contributes to the increase in the thickness of the mycelium section. (l) Aerial hyphae starting to segment their DNA in nucleoids; many of these are dead (bottom). (m, n) Sporulation phase; the mycelium reaches its maximum thickness (notice the scale) and a thin green layer of spores is visible on the surface of the medium (m). Under the phase-contrast microscope (n), the form of the hyphae cannot be seen. Arrows in pictures (m) and (n) show the edge of the mycelium layer.

hyphae are clearly visible within the mycelium, which suggests the presence of a membrane separating the cellular compartments. To detect the possible presence of cell walls in these locations, equivalent preparations were stained with WGA (see Methods). Cell walls crossed by septa are scarce (arrows in Fig. 3b, c), as compared with the septa made visible with FM 4-64 (Fig. 3a), indicating that most

membrane septa observed are not associated with a cell wall (or at least with a cell wall thick enough to be detected with WGA; see below). CFDA is an esterified fluorogenic substrate, widely used for assessing esterase activity in bacteria (Hoefel *et al.*, 2003). Once hydrolysed by non-specific intracellular esterases, the green-fluorescent carboxy-fluorescein group produced is retained in the cytoplasm

Fig. 2. Details of the initial developmental phases of *Streptomyces antibioticus* on surface cultures. Fluorescent staining, letters and numbers as in Fig. 1. (a) Young stained hyphae showing their compartmentalized nature (arrow). (b) Attenuation of the green fluorescence of live segments of the variegated mycelium (arrows). (c) A non-germinated spore (arrow) within a mass of variegated hyphae. (d, e) Live, intensely stained segments of the variegated mycelium previous to its growth as second mycelium (arrows); diffuse red material exiting the lysing segments can be seen. (f) Growth of the young second mycelium. The mycelium in samples (d–f) was spread, as described in Methods, to improve the visualization of the hyphae.

Fig. 3. Analysis of hyphae compartmentalization with several fluorescent indicators. Letters and numbers as in Fig. 1. (a) Mycelium stained with FM 4-64. (b, c) Hyphae stained with WGA. Arrows indicate the presence of cross cell walls. (d) Hyphae stained with CFDA and PI; compare with Fig. 1(e) and Fig. 2(a). Intensely stained green segments are visible within the live hyphae (arrows), indicating the presence of metabolically active compartments. (e, f) Hyphae stained simultaneously with CFDA and FM 4-64. Two separate images were obtained for CFDA (e) and FM 4-64 (f). Arrows point to equivalent positions in both pictures. (g, h) Hyphae stained with rhodamine-123 (red), PI (violet) and SYTO 9 (green). Two separate images were obtained for rhodamine-123 and PI (g) and for SYTO 9 and PI (h). Arrows indicate segments stained simultaneously with rhodamine-123 and SYTO 9, confirming their active, viable status.

(Darzynkiewicz *et al.*, 1992). This allows the use of this compound to directly detect viable and active bacterial cells. Fig. 3(d) show hyphae stained with both CFDA and PI. As with the SYTO 9/PI stain, a variegated morphology is clearly visible, once more indicating the compartmentalized nature of the mycelium. Remarkably, intense green spots are visible inside the hyphae that do not present a variegated appearance (arrows in Fig. 3d). These points indicate the presence of areas with more enzyme activity within the live, compartmentalized mycelium. Staining with CFDA and FM 4-64 simultaneously showed that these areas of activity are delimited by membranes, further demonstrating the compartmentalized nature of these hyphae (Fig. 3e, f). Fig. 3(g) shows hyphae stained with the membrane-potential indicator rhodamine-123 (red) and PI (shown

here in violet to facilitate their visualization). The same field stained with SYTO 9 (green) and with PI (also shown in violet) can be seen in Fig. 3(h). As expected, the viable segments, stained with both rhodamine-123 and SYTO 9, have an intact membrane potential, in contrast with the dead, PI-stained compartments. The results show that the variegated first mycelium is formed by segments that stain specifically with CFDA, rhodamine-123 and SYTO 9, separated by segments that stain specifically with PI. Separation between these segments is delineated by a membrane with little, if any, associated cell wall material.

The physical nature of the transverse septum was analysed in more detail by electron microscopy observations (Fig. 4). As expected, two different septa could be seen: a few thick,

Fig. 4. Transmission electron micrographs of *Streptomyces antibioticus* hyphae at different developmental phases. Letters and numbers as in Fig. 1. (a) A developed, viable hypha showing a characteristic vegetative septum, transverse to the longitudinal axis of the hypha (arrow); notice the contrast with the tenuous, curved structures visible in the following pictures. (b) A thin, oval membrane delimiting a cell compartment (arrow); associated membrane layers can be seen to the left (arrowhead). (c) Two thin, oval septa (arrows) delimiting three compartments, the central one showing abundant membrane formations. (d) A compartment delimited by an oval, single-membrane septum (arrow); membrane arrays are observed in the next hyphal segment showing an advanced lytic process. (e) Hyphal segments showing an advanced fragmentation process. (f, g) Completion of the first death round; live segments with a denser cytoplasm (arrows) remain among dead compartments (f). An overview of some of the fields (g) shows complete disorganization of the cells and extensive dismantling of the cell walls (arrowhead).

sporadic straight transverse septa (Fig. 4a), which correspond to the previously described vegetative mycelium septa (DeJong & McCoy, 1966), and other more frequent, thinner, less-rigid septa, which delimit irregular, rounded compartments (Fig. 4b–e). Electron microscopy analysis also revealed the presence of massive intracytoplasmic membrane arrays associated with the latter septa (Fig. 4b, d). An extensive degradation of the cell wall is seen in some of the segments (Fig. 4d–f). At later growth stages, numerous empty, disorganized dead segments are visible in some of the fields (Fig. 4g). Segments with a dense cytoplasm, which probably correspond to the live compartments observed using CLSM (Fig. 2d, e), are visible among these (Fig. 4g).

DISCUSSION

We have characterized two death rounds in *S. antibioticus* ATCC 11891: the first takes place very soon after the germination of the spores and affects the young compartmentalized mycelium, whilst the second, starting at about 25 h, affects the mycelium developed from the viable segments of the variegated hyphae. These observations rely on the fact that cells stained with PI have compromised membranes and are, consequently, not viable (Miller & Quarles, 1990; Lloyd & Hayes, 1995; Bunthof *et al.*, 1999, 2001; Haugland, 2002). This has been also documented with bacterial endospores (Laflamme *et al.*, 2004). The suitability of the SYTO 9/PI stain for testing the viability of *Streptomyces* has been analysed previously in liquid cultures (Fernandez & Sanchez, 2001), in which hyphae subjected to lethal artificial treatments were included as control. We never observed growth from a homogeneously PI-stained suspension of mycelium (A. Manteca and J. Sánchez, unpublished results). All experiments performed in the present work to corroborate the SYTO9/IP results, including the use of alternative fluorescent indicators such as CFDA/PI (Fig. 3d) or rhodamine/PI (Fig. 3g), and electron microscopy analyses (Fig. 4c–f), confirm its reliability for analysing development under surface-culture conditions. The present work also showed that most germinated hyphae under these conditions lost their membrane permeability at an early stage, although some of the spores emitted a germ tube that remained fully viable (arrow in Fig. 1c, and Fig. 1d). In contrast, germinated hyphae in submerged conditions appear mostly viable (data not shown). The visualization of both types of hyphae by green and red staining in the same microscope field (Fig. 1c) is further evidence of the reliability of the vital staining technique used here. In addition, the ‘variegated’ appearance of the compartmentalized young mycelium depends on environmental conditions. If a very diluted inoculum is used, in such a way that growth of individual colonies is obtained, the alternation of green (live) and red (dead) segments can not be seen until a high-density mycelial mass is reached (A. Manteca and J. Sánchez, unpublished results). Finally, the biochemical analysis with several death markers has established the existence of the early death round and the

lysis and degradation of the main cellular macromolecules during the death processes (Manteca *et al.*, 2005).

The second death round described in this work is equivalent to the death process proposed to occur in non-sporulating aerial hyphae of *S. coelicolor* by Wildermuth (1970), as well as in *S. antibioticus* ETH7451 surface cultures, a strain which grows faster and also sporulates in submerged conditions (Novella *et al.*, 1992; Fernandez & Sanchez, 2002). This is also likely to be the same death process reported by other authors in the strain used by us (Miguel *et al.*, 1999). In contrast, the early first death round affecting the compartmentalized young mycelium and the evidence for the presence of such compartmentalization have not been previously described, probably due to their ephemeral existence. It is noteworthy that this compartmentalization was detected with different fluorescence indicators and by electron microscopy. The intrinsic properties of the SYTO 9/PI fluorescent colourants used (Haugland, 2002) imply that the corresponding segments are separated by at least a membrane (see below). Intriguingly, the red-fluorescent compound FM 4-64, a membrane-specific dye previously used in *Streptomyces* (Grantcharova *et al.*, 2003), stained some zones of the *S. antibioticus* compartmentalized hyphae more intensely than others (Fig. 3a, f), a phenomenon also reported in *Escherichia coli* (Fishov & Woldringh, 1999). As in the latter bacterium, the intense red-fluorescent regions in *Streptomyces* may be explained by the presence of membrane domains with greater affinity for the dye (Fishov & Woldringh, 1999). WGA staining and electronic microscopy analysis revealed that these compartments lack any cellular walls detectable by these approaches.

If the density of hyphae is high at a particular point on the plate, the consumption of an essential nutrient(s) will overcome the flow of these substances through the agar. This could motivate the establishment of local starvation conditions in the microenvironment close to the hypha, which may lead to stress responses (Kelemen *et al.*, 2001). Gradients of oxygen and free radical concentration (Hahn *et al.*, 2002; van Keulen *et al.*, 2003) could have a similar effect. The metabolic activity of an isolated hypha or of a less concentrated group of hyphae would provoke a lesser disturbance of the surrounding medium. The compartmentalization of the mycelium and the survival capacity of some of the cellular segments jointly allow the bacteria to face up to these critical situations and would constitute a good adaptive response in their natural soil habitat. The phenomenon evokes that of ‘persistence’ within bacterial populations, in which the suicide programme is disabled in a fraction of cells when confronted with potentially lethal damage (Lewis, 2000). As yet, we do not know the mechanisms underlying the outstanding capacity of the first mycelium to partially inactivate a fraction of cells. An interesting possibility is the presence in *Streptomyces* of a kind of determining mechanism, such as that reported in *Bacillus subtilis*, in which the cells that have entered the pathway to sporulate produce and export a killing factor

that causes sister cells to lyse (Gonzalez-Pastor *et al.*, 2003). Nutrient replenishment/depletion effects in the agar medium may analogously motivate the second death round. In this case, however, death affects the much longer segments of the unseptated hyphae that form the aerial mycelium.

All studies to date describing the differentiation and developmental cycle of *Streptomyces* describe a fully viable substrate mycelium grown in culture medium, from which a reproductive (aerial) mycelium emerges (Wildermuth, 1970; Kalakoutsos & Agre, 1976; Mendez *et al.*, 1985; Chater, 1989; Hodgson, 1992). There is also consensus that the substrate mycelium and pre-sporulating aerial mycelium are syncytia with sporadic septa (Hodgson, 1992; Chater, 1993; Chater & Losick, 1997). The substrate mycelium in *S. antibioticus* ATCC 11891 would be the mycelium formed from spores and developed until around 35 h cultivation at 30 °C. The formation of the aerial mycelium would commence from this point on, and at around 60 h cultivation the sporulation process would begin (Mendez *et al.*, 1985; Miguelez *et al.*, 1999). In our work, it seems clear that a compartmentalized mycelium is formed after the germination of spores. This mycelium should not be considered as substrate mycelium or aerial mycelium, as it is non-syncytial in nature. The viable mycelium developed from viable segments of the so-called variegated hyphae is already a syncytium (not shown), and in consequence it would correspond to the substrate mycelium described by other authors, lasting until about 35 h, whereas at later times it would correspond to the aerial mycelium. Thus, the former designations 'substrate' and 'aerial' are not strictly applicable to the variegated mycelium or to the second mycelium described in our work.

The features described in this study are not a striking peculiarity of the species and strain used, as they have been detected in all *Streptomyces* analysed so far, including the genetically well-characterized species *S. coelicolor* (A. Manteca and J. Sánchez, unpublished results). It has been proposed that micro-organisms, and especially those that present a complex developmental life, such as *Streptomyces*, *Bacillus*, *Anabaena*, *Caulobacter*, *Rhizobium* and the myxobacteria, have programmed cell-death mechanisms and/or genes which could be considered to be the phylogenetic precursors of eukaryotic programmed cell death (Yarmolinsky, 1995; Hochman, 1997; Aravind *et al.*, 1999; Koonin & Aravind, 2002; this study). Kinase domains, similar to those from eukaryotic signal domains, are present in the gene products of the *Streptomyces* genome (Zhang, 1996; Aravind *et al.*, 1999; Elizarov & Danilenko, 2001; Petrickova & Petricek, 2003), some of which are membrane associated and calcium dependent (Elizarov & Danilenko, 2001). Moreover, *Streptomyces* harbours ATPases of the apoptotic type (Aravind *et al.*, 1999; Koonin & Aravind, 2002), which are typical components of eukaryotic signalling systems and, in particular, of the apoptotic system. The study of the function of these hypothetical signals and

effectors in the *Streptomyces* cell death processes described here will certainly constitute an important field of investigation in the future.

ACKNOWLEDGEMENTS

We wish to express our thanks to Professor B. A. Connolly, School of Cell and Molecular Biosciences, University of Newcastle, for his helpful suggestions with respect to the study. We also wish to thank Angel Martínez Nistal, Image Processing and Analysis Service of the University of Oviedo, for his indispensable assistance with the confocal microscope, José Manuel López García, Departamento de Morfología y Biología Celular, Carolina Alonso, Departamento de Biología Funcional, Universidad de Oviedo, for helping with the electron microscopy samples, and Paul Barnes for proof-reading the text. This research was funded by grant BIO2004-06089 from the DGI, Subdirección General de Proyectos de Investigación, MEC, Spain.

REFERENCES

- Aravind, L., Dixit, V. M. & Koonin, E. V. (1999). The domains of death: evolution of the apoptosis machinery. *Trends Biochem Sci* **24**, 47–53.
- Braña, A. F., Mendez, C., Diaz, L. A., Manzanal, B. A. & Hardisson, C. (1986). Glycogen and trehalose accumulation during colony development in *Streptomyces*. *J Gen Microbiol* **132**, 1319–1326.
- Bunthof, C. J., Sabina van den Braak, S., Breeuwer, P., Rombouts, F. M. & Abee, T. (1999). Rapid fluorescence assessment of the viability of stressed *Lactococcus lactis*. *Appl Environ Microbiol* **65**, 3681–3689.
- Bunthof, C. J., van Schalkwijk, S., Meijer, W., Abee, T. & Hugenholtz, J. (2001). Fluorescent method for monitoring cheese starter permeabilization and lysis. *Appl Environ Microbiol* **67**, 4264–4271.
- Chater, K. F. (1984). Morphological and physiological differentiation in *Streptomyces*. In *Microbial Development* (Monographic Series 16), pp. 89–115. Edited by R. Losick & L. Shapiro. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Chater, K. F. (1989). Aspects of multicellular differentiation in *Streptomyces coelicolor* A3(2). In *Genetics and Molecular Biology of Industrial Microorganisms*, pp. 99–107. Edited by C. L. Hershberger, S. W. Queener & G. Hegeman. Washington, DC: American Society for Microbiology.
- Chater, K. F. (1993). Genetics of differentiation in *Streptomyces*. *Ann Rev Microbiol* **47**, 685–713.
- Chater, K. F. & Losick, R. (1997). The mycelial life-style of *Streptomyces coelicolor* A(3)2 and its relatives. In *Bacteria as Multicellular Organisms*, pp. 149–182. Edited by J. H. Shapiro & M. Dworkin. New York: Oxford University Press.
- Darzynkiewicz, Z., Bruno, S., Del Bino, G., Gorczyca, W., Hotz, M. A., Lassota, P. & Traganos, F. (1992). Features of apoptotic cells measured by flow cytometry. *Cytometry* **13**, 795–808.
- DeJong, P. J. & McCoy, E. (1966). Qualitative analysis of vegetative cell walls and spore walls of some representative species of *Streptomyces*. *Can J Microbiol* **12**, 985–994.
- Elizarov, S. M. & Danilenko, V. N. (2001). Multiple phosphorylation of membrane-associated calcium-dependent protein serine/threonine kinase in *Streptomyces fradiae*. *FEMS Microbiol Lett* **202**, 135–138.
- Fernandez, M. & Sanchez, J. (2001). Viability staining and terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling of the mycelium in submerged cultures of *Streptomyces antibioticus* ETH7451. *J Microbiol Methods* **47**, 293–298.

- Fernandez, M. & Sanchez, J. (2002). Nuclease activities and cell death processes associated with the development on surface cultures of *Streptomyces antibioticus* ETH7451. *Microbiology* **148**, 405–412.
- Fishov, I. & Woldringh, C. L. (1999). Visualization of membrane domains in *Escherichia coli*. *Mol Microbiol* **32**, 1166–1172.
- Gonzalez-Pastor, J. E., Hobbs, E. C. & Losick, R. (2003). Cannibalism by sporulating bacteria. *Science* **301**, 510–513.
- Granozzi, C., Billea, R., Passantino, R., Sollazzo, M. & Puglia, A. M. (1990). A breakdown in macromolecular synthesis preceding differentiation in *Streptomyces coelicolor* A3(2). *J Gen Microbiol* **136**, 713–716.
- Grantcharova, N., Ubhayasekera, W., Mowbray, S. L., McCormick, J. R. & Flardh, K. (2003). A missense mutation in *ftsZ* differentially affects vegetative and developmentally controlled cell division in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **47**, 645–656.
- Hahn, J. S., Oh, S. Y. & Roe, J. H. (2002). Role of OxyR as a peroxide-sensing positive regulator in *Streptomyces coelicolor* A3(2). *J Bacteriol* **184**, 5214–5222.
- Haugland, R. P. (2002). Nucleic acid detection and genomics technology. In *Handbook of Fluorescent Probes and Research Chemicals*, 9th edn, Chapter 8. Edited by J. Gregory. Eugene, OR: Molecular Probes, Inc.
- Hochman, A. (1997). Programmed cell death in prokaryotes. *Crit Rev Microbiol* **23**, 207–214.
- Hodgson, D. A. (1992). Differentiation in Actinomycetes. In *Prokaryotic Structure and Function: a New Perspective* (Society for General Microbiology Symposium no. 47), pp. 407–440. Edited by S. Mohan, C. Dow & J. A. Cole. Cambridge: Cambridge University Press.
- Hoefel, D., Grooby, W. L., Monis, P. T., Andrews, S. & Saint, C. P. (2003). A comparative study of carboxyfluorescein diacetate and carboxyfluorescein diacetate succinimidyl ester as indicators of bacterial activity. *J Microbiol Methods* **52**, 379–388.
- Hopwood, D. A. & Glauert, A. M. (1961). Electron microscope observations on the surface structures of *Streptomyces violaceoruber*. *J Gen Microbiol* **26**, 325–330.
- Kalakoutskii, L. V. & Agre, N. S. (1976). Comparative aspects of development and differentiation in actinomycetes. *Bacteriol Rev* **40**, 469–524.
- Kelemen, G. H., Viollier, P. H., Tenor, J., Marri, L., Buttner, M. J. & Thompson, C. J. (2001). A connection between stress and development in the multicellular prokaryote *Streptomyces coelicolor* A3(2). *Mol Microbiol* **40**, 804–814.
- Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. & Hopwood, D. A. (2000). Growth and Preservation of *Streptomyces*. In *Practical Streptomyces Genetics*, Chapter 2, pp. 43–61. Norwich, UK: The John Innes Foundation.
- Koonin, E. V. & Aravind, L. (2002). Origin and evolution of eukaryotic apoptosis: the bacterial connection. *Cell Death Differ* **9**, 394–404.
- Laflamme, C., Lavigne, S., Ho, J. & Duchaine, C. (2004). Assessment of bacterial endospore viability with fluorescent dyes. *J Appl Microbiol* **96**, 684–692.
- Lewis, K. (2000). Programmed death in bacteria. *Microbiol Mol Biol Rev* **64**, 503–514.
- Lloyd, D. & Hayes, A. J. (1995). Vigour, vitality and viability of microorganisms. *FEMS Microbiol Lett* **133**, 1–7.
- Lopez-Amoros, R., Comas, J. & Vives-Rego, J. (1995). Flow cytometric assessment of *Escherichia coli* and *Salmonella typhimurium* starvation-survival in seawater using rhodamine 123, propidium iodide, and oxonol. *Appl Environ Microbiol* **61**, 2521–2526.
- Manteca, A., Fernández, M. & Sánchez, J. (2005). Cytological and biochemical evidence for an early cell dismantling event in surface cultures of *Streptomyces antibioticus*. *Res Microbiol*. In press.
- Mendez, C., Braña, A. F., Manzanal, M. B. & Hardisson, C. (1985). Role of substrate mycelium in colony development in *Streptomyces*. *Can J Microbiol* **31**, 446–450.
- Miguel, E. M., Hardisson, C. & Manzanal, M. B. (1999). Hyphal death during colony development in *Streptomyces antibioticus*: morphological evidence for the existence of a process of cell deletion equivalent to apoptosis in a multicellular prokaryote. *J Cell Biol* **145**, 515–525.
- Miller, J. S. & Quarles, J. M. (1990). Flow cytometric identification of microorganisms by dual staining with FITC and PI. *Cytometry* **11**, 667–675.
- Novella, I. S., Barbes, C. & Sanchez, J. (1992). Sporulation of *Streptomyces antibioticus* ETHZ 7451 in submerged culture. *Can J Microbiol* **38**, 769–773.
- Okanishi, M., Suzuki, K. & Umezawa, H. (1974). Formation and reversion of Streptomyces protoplasts: cultural condition and morphological study. *J Gen Microbiol* **80**, 389–400.
- Petrickova, K. & Petricek, M. (2003). Eukaryotic-type protein kinases in *Streptomyces coelicolor*: variations on a common theme. *Microbiology* **149**, 1609–1621.
- Pogliano, J., Osborne, N., Sharp, M. D., Abanes-De Mello, A., Perez, A., Sun, Y. L. & Pogliano, K. (1999). A vital stain for studying membrane dynamics in bacteria: a novel mechanism controlling septation during *Bacillus subtilis* sporulation. *Mol Microbiol* **31**, 1149–1159.
- Rice, K. C. & Bayles, K. W. (2003). Death's toolbox: examining the molecular components of bacterial programmed cell death. *Mol Microbiol* **50**, 729–738.
- Schwedock, J., McCormick, J. R., Angert, E. R., Nodwell, J. R. & Losick, R. (1997). Assembly of the cell division protein FtsZ into ladder-like structures in the aerial hyphae of *Streptomyces coelicolor*. *Mol Microbiol* **25**, 847–858.
- Sun, Q. & Margolin, W. (1998). FtsZ dynamics during the division cycle of live *Escherichia coli* cells. *J Bacteriol* **180**, 2050–2056.
- van Keulen, G., Jonkers, H. M., Claessen, D., Dijkhuizen, L. & Wosten, H. A. (2003). Differentiation and anaerobiosis in standing liquid cultures of *Streptomyces coelicolor*. *J Bacteriol* **185**, 1455–1458.
- Vida, T. A. & Emr, S. D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J Cell Biol* **128**, 779–792.
- Waksman, S. A. (1967). The genus *Streptomyces*. In *The Actinomycetes. A Summary of Current Knowledge*, Chapter 9, pp. 101–112. New York: The Ronald Press Co.
- Wildermuth, H. (1970). Development and organization of the aerial mycelium in *Streptomyces coelicolor*. *J Gen Microbiol* **60**, 43–50.
- Yarmolinsky, M. B. (1995). Programmed cell death in bacterial populations. *Science* **267**, 836–837.
- Zhang, C. C. (1996). Bacterial signalling involving eukaryotic-type protein kinases. *Mol Microbiol* **20**, 9–15.