Amplification of a *Streptomyces lividans* 4.3 kb DNA element causes overproduction of a novel hypha- and vesicle-associated protein

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**INTRODUCTION**

Streptomyces are Gram-positive soil bacteria that grow as substrate mycelia. Upon nutrient depletion the substrate mycelium differentiates into aerial mycelium and spores. *Streptomyces* strains produce a wide range of pigments, antibiotics, cytostatics and fungicides. In addition they degrade many macromolecules, including lignocellulose, chitin, starch and xylans (Kutzner, 1981). Several studies have demonstrated that genes encoding characteristics such as resistance to antibiotics, antibiotic production, melanin formation and A-factor synthesis can be lost at high frequencies (for reviews see Schrempf, 1985; Schrempf et al., 1989; Schneider et al., 1993; Leblond & Decaris, 1994).

The genomes of streptomycetes range in size between 6.5 and 8 Mbp (Gladek & Zakrzewska, 1984; Leblond et al., 1993), and recent evidence suggests that they are linear (Lin et al., 1993). Deletions of various sizes are encountered, and consist of continuous stretches of DNA that may be several hundred to about 1000 kb long (Schrempf et al., 1989; Dary et al., 1993). *Streptomyces coelicolor* A3(2) and its close relative *Streptomyces lividans* 66 have the so-called ‘silent’ arc centred at 3 o’clock in the conventionally drawn circular map of the chromosome. Most of the characterized genetic markers are absent from this region (Hopwood et al., 1985). Our studies have indicated that the unstable genes encoding chloramphenicol (Dittrich et al., 1991) and tetracycline (Kessler et al., 1989) resistance, and argininosuccinate synthase (Betzler et al., 1987) are located within the ‘silent’ region. In a corresponding region of the *Streptomyces ambofaciens* genome, some unstable genes of yet unexplored function were found (Dary et al., 1993).

Besides deletions, amplifications of DNA stretches of varying sizes occur in some streptomycetes. They may be the result of the selection for overproduction of the α-amylase inhibitor tendamistat (*Streptomyces tendae*, Koller, 1986), or for high levels of resistance to antibiotics (chloramphenicol in *S. lividans*, Dittrich et al., 1991, or spectinomycin in *Streptomyces achromogenes*, Hornemann et al., 1986). However, most amplifications occur without any apparent selection, and therefore genes encoded by amplified DNA are frequently of unknown function.

*S. lividans* 66 displays low resistance against chloramphenicol, tetracycline, and some other antibiotics (Schrempf, 1985). Chloramphenicol-sensitive mutants arise at frequencies of about $10^{-3}$ (Altenbuchner & Cullum, 1985) and carry a chromosomal deletion of the

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gene encoding efflux of chloramphenicol (cml) and neighbouring DNA regions (Dittrich et al., 1991). These mutants often delete the argG (argininosuccinate synthase) gene. This deletion is accompanied by an amplification of the 6.8 kb amplifiable DNA unit (AUD) (Betzler et al., 1987; Dyson & Schrempf, 1987), but further selection for resistance to chloramphenicol (Cml) yields variants no longer resistant to tetracycline (Tet). The Tet-sensitive strains carry large chromosomal deletions that include a resistance gene encoding a protein homologous to the Tet(M)-Tet(0) group of tetracycline resistance proteins (Schrempf et al., 1989; Kessler et al., 1989; Dittrich & Schrempf, 1992). The Cml-resistant and Tet-sensitive mutant TKIII-10T carries a 4.3 kb AUD in a tandem array of several hundred copies (Dyson et al., 1986; Schrempf et al., 1989).

In this report we characterize the 4.3 kb AUD and compare its sequence with the corresponding amplified DNA sequence (ADS). In addition, we have identified a novel 23 kDa protein encoded by the AUD and ADS; this protein is associated with the hyphae, and with newly discovered hyphal bulges and vesicles in the S. lividans TKIII-10T variant.

METHODS

Media, culture conditions, bacterial strains and plasmids. S. lividans 66 wild-type was provided by D. A. Hopwood (John Innes Institute, Norwich, UK). Variants derived from this strain had been described earlier (Dyson et al., 1986; Kessler et al., 1989). Plasmids pUC8, pUC18 (Yanisch-Perron et al., 1985), and pUS18 (constructed in our laboratory by inserting an additional SstII–NotI–SfiI–SpeI linker into the pUC18 BamHI site; Dittrich et al., 1991) were used as cloning vectors for DNA sequence analysis. Escherichia coli strains JM83 (Yanisch-Perron et al., 1985) and K-12 F'–M15 (Rüther et al., 1981) served as hosts. Plasmids pT7-7 and pGFI-2 (Tabor & Richardson, 1985) were used for gene expression experiments. E. coli strains harbouring plasmids were grown in LB medium containing 100 μg ampicillin ml−1. Streptomyces strains were cultivated in minimal or complete medium (Dyson & Schrempf, 1987).

Isolation of DNA. Genomic DNA from Streptomyces strains was isolated as described previously (Hopwood et al., 1985). Plasmids were isolated from E. coli with a Qiagen plasmid kit.

In vitro manipulation of DNA and hybridizations. Recovery of DNA from agarose gels, ligation of DNA, preparation of competent E. coli cells and their transformation were described by Dyson & Schrempf (1987) and Sambrook et al. (1989).

DNA or RNA was transferred from gels to nylon membranes (Sambrook et al., 1989). DNA–DNA or RNA–DNA hybridizations were carried out using probes labelled with digoxigenin (Boehringer Mannheim).

Isolation of RNA. Total RNA from S. lividans was isolated according to the method of Alba et al. (1981) with the following alterations: mycelium from a 10 ml culture was washed once in TE buffer [10% (w/v) sucrose, 5 mM EDTA, 30 mM Tris/HC1, pH 8.0] and twice in 50 mM sodium acetate, 10 mM EDTA, pH 5.0. One volume of phenol (saturated with 50 mM sodium acetate, 10 mM EDTA, pH 8.0) was added and the SDS concentration was adjusted to 1% (w/v). The mixture was incubated with vigorous shaking for 4 min at 65°C, chilled in a bath of dry ice and ethanol, and centrifuged for 2 min. The supernatant was extracted twice with phenol and once with chloroform. RNA was recovered by ethanol precipitation.

DNA sequencing. The 5.4 kb PstI–SalI fragment which contained the AUD and flanking regions, and the PstI fragment including the 4.3 kb ADS copy were sequenced by the dideoxy chain-termination technique (Sanger et al., 1977) with Sequenase (US Biochemical). Standard universal and reverse primers or specifically synthesized oligonucleotides were used for priming. Sequences were analysed with the programs GEMON (GBF, Braunschweig, Germany), GCwind (D. Shields, Dublin, Ireland), FASTA (Pearson & Lipman, 1988), and BLITZ (S. Sturrock & J. Collins, MPSrch version 1.5; Biocomputing Research Unit, University of Edinburgh, UK); the SWISS-PROT/EMBL database served for protein sequence comparisons.

Protein analysis. To isolate the 23 kDa protein, mycelium from S. lividans TKIII-10T was washed three times in TP buffer (20 mM Tris/HC1, pH 7.4, 0.1 mM PMSF). Combined wash fractions were precipitated with 90% (w/v) (NH4)2SO4 and pelleted at 20000 g. The resulting proteins were resuspended in TP buffer, dialysed for 16 h against the same buffer and stored in 50% (v/v) glycerol at −20°C.

Determination of the N-terminal sequence. Proteins were separated by SDS-PAGE, blotted onto a nylon membrane and stained with Amidoschwarz. The region of the membrane containing the overproduced 23 kDa protein was excised, and the corresponding amplified DNA fragment was excised from the E. coli plasmid pT7-7 vector, resulting in the plasmid pT7K1.

Transformants harbouring pT7K1 in addition to pGFI-2 were grown with 40 μg ampicillin ml−1 and 40 μg kanamycin ml−1 to OD600 = 0.5. A sample (0.2 ml) of the culture was washed twice in M9 minimal medium (Sambrook et al., 1989) and resuspended in 1 ml of the same medium containing all proteinic amino acids (each 0.01%) except cysteine and methionine, 20 μg thiamin ml−1 and 0.4% glucose. After growth for 60 min at 30°C, the culture was incubated at 42°C for 15 min. Rifampicin was added to a concentration of 200 μg ml−1 and incubation was extended for 10 min. The culture was shifted to 30°C for 20 min, and then 35S-methionine (10 μCi = 3·7·104 Bq) was added for 5 min. After centrifugation, cells were lysed in 120 μl lysis buffer [60 mM Tris/HC1, pH 6.8; 1% (w/v) SDS; 1% (w/v) β-mercaptoethanol; 10% (v/v) glycerol; 0.01% bromophen blue]. A 40 μl portion of this suspension was loaded on a 12.5% SDS-polyacrylamide gel. Alternatively, cells were grown in enriched media [2% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% NaCl; 0.2% glycerol; 50 mM KH2PO4, pH 7.2; 50 μg ampicillin and kanamycin ml−1] to OD600 = 1.5.

After the culture had been incubated at 42°C for 25 min, rifampicin was added to a final concentration of 200 μg ml−1 and cells were left at 35°C for 2 h. The cells from 100 μl culture were collected by centrifugation, washed twice in M9 medium, lysed with 40 μl lysis buffer, and loaded on a 12.5% SDS-polyacrylamide gel.

Isolation of inclusion bodies from E. coli. Inclusion bodies were isolated (Marston et al., 1984), resuspended in 5 M urea and dialysed against 10 mM Tris/HC1, pH 8.0, for 24 h. The
purity of the protein was analysed by PAGE, and its amount was determined by the Bradford (1976) method.

**Enrichment for bulbous elements.** S. lividans TKIII-10T was grown for 4 d in YEME medium containing 1% (w/v) galactose instead of glucose. Mycelia were washed in 20 mM potassium phosphate buffer, pH 7.2, and centrifuged for 2 min at 50 g to increase the content in the supernatant of mycelia containing bulbous structures. To the supernatant 1 mg pronase ml⁻¹ was added, and after 60 min cell debris was removed by low-speed centrifugation (2500 g for 10 min), and granules were sedimented at 50000 g.

**Tests for glycogen and trehalose.** Previously described enzymic methods were used to test for the presence of glycogen and trehalose (Schulze et al., 1993).

**Gas chromatography.** Granules extracted with chloroform were analysed by gas chromatography (courtesy of A. Steinbüchel, University of Göttingen, Germany).

**Production of antibodies and Western blot analysis.** Antisera were obtained by immunization of rabbits with the purified fusion protein mixed with Freund's complete and incomplete adjuvants. For immunodetection, proteins were transferred to nylon filters after SDS-PAGE. The blots were incubated in NaCl/phosphate buffer (136 mM NaCl, 2.6 mM KCl, 1.4 mM KH₂PO₄, 81 mM Na₂HPO₄, pH 7.1) containing a 1:5 x 10⁵ dilution of the antiserum, washed three times in NaCl/phosphate buffer, and incubated with alkaline-phosphatase-conjugated goat anti-rabbit IgG F(ab)₂ diluted 1:5000 in NaCl/phosphate buffer. Colour development was performed as described by West et al. (1990).

**Immunomicroscopical studies.** Coverslips were coated with poly-d-lysine (molecular mass 50 kDa) solution (50 µg ml⁻¹ in H₂O) and dried under a filtered air flow. Serial 1:10 dilutions of mycelia were dropped in the middle of coated coverslips, as described by Schnellmann et al. (1994). The coverslip was dried in a laminar flow hood, then placed in a small Petri dish and washed three times in buffer [1:4 M NaCl; 2.6 mM KCl; 1.4 mM KH₂PO₄; 81 mM Na₂HPO₄; 1% (w/v) blocking reagent (Boehringer Mannheim); pH 7.1]. A 1:100 dilution of the antiserum was added, and after 4 h the sample was again washed three times in buffer. Fluorescein-labelled secondary antibodies were added and allowed to react for 4 h. After three washes in buffer, the last wash without blocking reagent, fluorescence was visualized using UV light, Ilford HP5 film, and an Axiosvert microscope (Zeiss), or by confocal laser microscopy (Zeiss).

**Electron microscopy.** Thin sections of the growing hyphae (Schlochtermeier et al., 1992) were prepared and analysed with a Zeiss electron microscope 109. Photos were taken with Kodakith MPhll film (Kodak).

**RESULTS**

**Characteristics of AUD and ADS**

The chromosomal DNA of wild-type S. lividans contains one copy of the 4.3 kb AUD, which is amplified to several hundred tandem copies within the tetracycline-sensitive variant TKIII-10T. A mutant strain TKIII-10C arose by spontaneous deletion of this ADS (Dyson et al., 1986; Kessler et al., 1989). PsI digestion of total DNA from TKIII-10T generated large quantities of a 4.3 kb fragment, which was subsequently cloned in the E. coli vector pUC8 (Dyson et al., 1986). The resulting construct (pTK1) was used as a probe to screen a gene library in the Charon vector 35 (Loenen & Blatter, 1983) from the wild-type S. lividans DNA. DNAs from overlapping hybrid phage (Schrempf et al., 1989) containing the 4.3 kb PsI fragment (Fig. 1a), were mapped and subcloned.

The sequence corresponding to the wild-type AUD was determined and compared to the stretch cloned (in pTK1, see above) from the ADS. The AUD consists of 4317 bp and is not flanked by either direct or indirect repeats. In the AUD (Fig. 1b), a stretch containing the three ORFs orf-2 (654 bp), orf-3 (987 bp), and orf-4 (1029 bp) is flanked by orf-1 (729 bp) and the partially (156 bp) sequenced orf-5. Within the ADS of TKIII-10T, all of orf-2, orf-3, and orf-4 are amplified, whereas only the end of orf-1 (543 bp) is included; it is linked to 91 bp of orf-5 facing in the opposite direction.

**Analysis of the DNA sequence and the deduced proteins**

The nucleotide sequence of the 5424 bp PsI–SalI fragment containing the AUD does not correspond to any known sequence and comprises four complete ORFs. However, a small region (nucleotides 3511–3668, situated between orf-3 and orf-4) corresponds to a DNA fragment from the S. lividans genome, which had earlier been identified (in the course of shotgun cloning experiments, Forssmann & Jaurin, 1987) as a strong promoter during the late stage of growth.

The deduced proteins encoded by orf-1, orf-3 and the partially sequenced orf-5 have no significant amino acid identity with any known protein. orf-2 encodes a 23 kDa protein, the deduced amino acid sequence of which shares some similarity with several other deduced proteins of unknown function, including those encoded downstream of the folC gene in Lactobacillus casei (Toy & Bognar, 1990), in the trpS–dam region of E. coli (Lyngstadaas & Boye, SWISS-PROT/EMBL database), upstream of trpE of Pseudomonas putida (Essar et al., 1990), in the tnaB–bgI region of E. coli (Yieh, Burland et al., 1993), and in the genome of Haemophilus influenzae (CbbY, Schäferjohann et al., 1993; Yhfe, Fleischmann et al., 1995). It has been suggested that CbbY, CbbZ, Yhfe and Yieh form a family of proteins. CbbZ from Alcaligenes eutrophus (Schäferjohann et al., 1993) was recently identified as 2-phosphoglycolate phosphatase. A few amino acids from the deduced 23 kDa protein are found in two motifs (34 and 27 aa) within three deduced haloacetate dehalogenases from Pseudomonas putida (Schneider et al., 1991; Jones et al., 1992) and one dehalogenase from Moraxella sp. strain B (Kawasaki et al., 1992).

Of the 343 amino acid sequence deduced from orf-4, 26 and 25 % are identical with UDPgalactose 4-epimerases identified from S. lividans (Adams et al., 1988) and E. coli (Lemaire & Müller-Hill, 1986), respectively. Moreover, this includes an arrangement of amino acids suggested as a signature for ‘RED’ enzymes.
M. BETZLER, I. TLOLKA and H. SCHREMPF

Hpl7K 1
PK 1
ss PS ss PS ss PS
ADS
ss PS ss SP
WT
AUD
SA 1
1
I
SA 2
PB ss X PBBSB XS
AUD
Figrn
7. (a) Restriction maps of cloned S. lividans genome DNA. The inserts from the phage SA1 and SA2 containing the AUD from the wild-type (WT) were mapped with restriction enzymes. The relative locations of the cleavage sites were compared with those situated in genomic DNA from the mutant TKIII-1OT and from part of the ADS cloned in pTK1. pT7K1 is the construct that overexpresses the gene product of 062.

(b) Relative arrangement of the sequenced ORFs within the AUD. Abbreviations: B, BamHI; Bg, BglII; P, PstI; S, SalI; X, Xhol.

Fig. 7. (a) Restriction maps of cloned S. lividans genome DNA. The inserts from the phage SA1 and SA2 containing the AUD from the wild-type (WT) were mapped with restriction enzymes. The relative locations of the cleavage sites were compared with those situated in genomic DNA from the mutant TKIII-1OT and from part of the ADS cloned in pTK1. pT7K1 is the construct that overexpresses the gene product of 062. (b) Relative arrangement of the sequenced ORFs within the AUD. Abbreviations: B, BamHI; Bg, BglII; P, PstI; S, SalI; X, Xhol.

Identification of a 23 kDa protein and its corresponding gene

To detect any possible gene products encoded by the amplified DNA, total proteins were isolated from mycelia of the wild-type and the variants, and separated by SDS-PAGE. A considerable quantity of the 23 kDa protein was discovered in the strain TKIII-1OT only when mycelia had been shaken for 5 d in YEME medium. Additional studies revealed that several proteins, including a 23 kDa protein, could be released from the pellet of older cultures (grown for 5–6 d) by consecutive washings with 20 mM Tris/HCl containing PMSF. The pellet consisted of intact and possibly leaky substrate hyphae and newly identified vesicles (see section below). The concentrated protein-containing suspension was separated by SDS-PAGE (Fig. 3). The sequence of ten amino acids at the N-terminus of the 23 kDa protein was determined and found to correspond to the ten N-terminal amino acids of the protein encoded by orf-2. This result in addition to the analysis of a hydrophobicity plot (data not shown) of the deduced protein, indicated the lack of a signal peptide.

The G+C content of orf-2 (654 bp) is 76 mol% and thus slightly higher than that of most other known S. lividans genes. A possible RBS (AGGA) is located 7 bp upstream of the ATG start codon (Fig. 2). orf-2 could encode a protein (218 aa) with a calculated molecular mass of 22.8 kDa, which agrees with the apparent molecular mass of the mature 23 kDa protein.

Overproduction of a truncated 23 kDa protein in E. coli

In E. coli transformants carrying pTK1, the gene encoding the 23 kDa protein was not transcribed; therefore the SalI–PstI fragment of pTK1 was placed under the control of the T7 promoter (in the plasmid pT7-7). In the resulting construct pT7K1 (Fig. 1a), the oligonucleotide corresponding to 13 N-terminal amino acids of the 23 kDa protein was replaced with 30 nucleotides of the T7 gene 10. The Shine–Dalgarno and T7 promoter region of gene 10 were thereby linked to the truncated gene. After induction, E. coli harbouring pT7K1 produced inclusion bodies, from which the 23 kDa protein was obtained after renaturation. Larger quantities of the protein were purified and used to raise antibodies (Fig. 3).

Characteristic features of the strains

In contrast to the wild-type, the variants TKIII-10T and TKIII-10C did not sporulate. In submerged cultures, the wild-type formed relatively long hyphae. The strain TKIII-10T (carrying the ADS) produced shorter hyphae which contained oval bulbous structures after 2 d of cultivation. In the following days, these developed into larger, round vesicle-like elements located predominantly at the hyphal apices. The mycelium of the cultures broke up into fragments of differing length. TKIII-10C, carrying a deletion of the 4.3 kb AUD, lacked these structures (Figs 4 and 5). The number of vesicles was about 3–4-fold higher if glucose in the YEME medium was replaced with galactose at the same concentration.

Mild heat treatment (~50 °C), followed by plating on agar, did not result in the generation of mycelia from the round elements; thus the possibility that they constitute sporangia-like structures could be excluded. Failure to detect spore-containing sporangia by transmission electron microscopy supported this finding (Fig. 5f). In cross-sections of the bulbous structures densely packed storage-like material was detected. Additional tests indicated that the storage-like material within the vesicles is not trehalose, glycogen, butyric or hydroxybutyric acid or a dominant lipid type. Analysis by SDS-PAGE excluded that the storage-like material contained detectable levels of the 23 kDa protein.
DNA amplification in *Streptomyces lividans*

**Fig. 2.** Nucleotide and deduced amino acid sequence of the 5424 bp *PstI-SalI* fragment. Potential RBSs (underlined) and inverted repeats (inverse arrows) are indicated. Asterisks denote translational termination codons. The 4317 bp DNA region amplified in TKIII-1OT is shown in parentheses.

**Fig. 3.** Identification of the 23 kDa protein. The *S. lividans* strains WT (lanes 1 and 2), TKIII-1OC (lanes 3 and 4), and TKIII-10T (lanes 5 and 6) were cultivated in YEME medium for 4 d (lanes 1, 3, 5) and 6 d (lanes 2, 4, 6), respectively. Proteins were released from mycelia as described in Methods. After blotting, the 23 kDa protein (lane 7, control) was detected by Western blot analysis with antibodies raised against the truncated 23 kDa form.

Localization of the 23 kDa protein

TKIII-10T synthesized large quantities of a transcript (~800 nucleotides) hybridizing to orf-2 (data not shown). Mycelia of the three strains were treated with primary antibodies (raised against the 23 kDa protein) and subsequently with fluorescein-labelled secondary antibodies. Investigations under UV light using a phase or confocal laser microscope revealed the 23 kDa protein could be detected on the surfaces of the substrate hyphae (grown on agar or in liquid culture for 1–6 d) of the wild-type (Fig. 6a) and of strain TKIII-1OT. The bulges and vesicles of TKIII-10T are covered with relatively large quantities of the protein (Fig. 6c). In contrast, hyphae from TKIII-1OC lack the protein (Fig. 6b). Controls conducted with preimmune serum or with secondary antibodies only did not lead to labelling of the mycelia.

**DISCUSSION**

The 43 kb amplifiable DNA unit (AUD) from *S. lividans* contains four open reading frames. One of them, orf-2, encodes a novel 23 kDa protein which associates with...
the substrate mycelium of the wild-type *S. lividans*. It is the first of its type and shows no sequence similarity to the few well-studied surface proteins of other bacteria including the M proteins from streptococci (Scott & Caparon, 1993) or surface (S)-layer proteins from various bacteria. Preliminary data suggest that the 23 kDa protein is abundant among streptomycetes and may play a general role, which remains to be elucidated.

The 23 kDa protein is absent from mycelia in the strain TKIII-10C carrying a deletion of the AUD. Amplification of the 4.3 kb AUD leads to disruption of *orf-1* and its linkage to a small residual part of *orf-5*, facing in the opposite direction. This does not lead to the formation of another ORF. The arrangement of *orf-2*, *orf-3* and *orf-4* remains unchanged after amplification. The 23 kDa protein was overproduced in the variant TKIII-10T containing the ADS, and in this strain, in addition to the vegetative substrate hyphae, larger amounts are associated with the bulbous structures. In the course of their early development, the bulges are found within the substrate mycelia; at later stages of growth, vesicle-like structures dominate at the ends of the hyphae. As visualized by transmission electron microscopy, the bulges and vesicles contain densely packed material which appears to be storage material. Its nature is unknown, but trehalose (Schulze et al., 1995); glycogen (Preiss, 1984), polyhydroxybutyrate (Steinbüchel & Schlegel, 1991), or a predominant type of lipid can be excluded as possibilities. In addition to glycogen, triacylglycerol has been found recently within *Streptomyces* species, including *S. lividans* (Olukoshi & Packter, 1994). Whether triacylglycerol is formed in large quantities by *S. lividans* TKIII-10T remains to be elucidated.

The proteins encoded by *orf-3* and *orf-4* have not been identified up to now but it is possible that they are enzymes involved in forming the storage material detected within the bulges and vesicles. The protein deduced from *orf-4* shares a motif with UDPglucose 4-epimerases, with GalE from various bacteria, and with ExoB from *Sinorhizobium* (*Rhizobium*) *meliloti* (Buendia et al., 1991). The promoter of *orf-4* had previously been identified as being expressed in the late stages of growth (Forsman & Jaurin, 1987). This agrees with our finding that the bulges and vesicles are found during this stage.

The morphological characteristics of the *S. lividans* variant TKIII-10T resemble those of *Intrasporangium calvum*, which forms branching substrate hyphae and no aerial mycelia. Oval and lemon-shaped vesicles
DNA amplification in *Streptomyces lividans*... 

**Fig. 5.** Microscopy of *S. lividans* strains. Mycelia grown for 5 d from the wild-type (a) and the variant TKIII-10T (d) were examined by scanning electron microscopy. Hyphae from the wild-type (b), TKIII-10C (c), as well as vesicles containing hyphae from TKIII-10T (e) were analysed by phase microscopy under visible light. (f) Transmission electron microscopy of a thin section through a vesicle from TKIII-10T. (a)–(e) Bar, 10 μm; (f) bar, 1 μm.

**develop** intercalarily and/or at the hyphal apices (Kalakoutskii *et al.*, 1967). The vesicles are abundant in older cultures. It was postulated that they are the result of hyphal swelling and gradual disorganization (Lechevalier & Lechevalier, 1969). *Intrasporangium calvum* has a G+C content of 70 mol% (Kalakoutskii *et al.*, 1967). It has chemotype I (LL-diamino-pimelic acid and glycine) cell walls, and has sometimes been assigned to the family *Streptomycetaceae* (Pridham & Tresner, 1974) on the basis of morphology, phospholipid composition (Lechevalier *et al.*, 1977), fatty acids (Kutzner, 1981), and susceptibility to genus-specific phage (Prauser & Falta, 1968; Wellington & Williams, 1981). However numerous taxonomic data point to a separate position for *Intrasporangium*. The formation of extensive mycelia and vesicles, as well as its fatty acid spectrum (Kutzner, 1981) and phospholipid composition distinguish *Intrasporangium* from nocardioforms and coryneforms (Lechevalier *et al.*, 1977). Our results clearly indicate the plasticity of the morphology within variants of one *Streptomyces* species. Molecular studies are required to clarify the present controversial relationship between *Intrasporangium* and other genera of the order Actinomycetales.

The *S. lividans* wild-type chromosome harbours only one copy of the 4·3 kb AUD (Schrempf *et al.*, 1989), which is situated in the 'silent' region (Schrempf *et al.*, 1989, and unpublished results). The 4·3 kb AUD is not flanked by repeats and its amplification has been observed only in *S. lividans* TKIII-10T. Other examples of type 1 AUD lacking repeated sequences or carrying only short microhomologies within very short imperfect repeats have been detected within *Streptomyces glaucescens* (Häusler *et al.*, 1989) and *S. ambofaciens* (Leblond *et al.*, 1990). In contrast, two tandem copies of AUD1 (consisting of three 1 kb repeats which flank two 4·7 kb parts) reside in the ‘silent’ region of *S. lividans* wild-type chromosome (Altenbuchner & Cullum, 1985; Schrempf, 1985; Dyson & Schrempf, 1987; Piendl *et al.*, 1994). Thus, AUD1 is a member of the class II AUDs (Hütter & Eckhardt, 1988) because it has long repeats and leads to reproducible tandem amplifications. Recent sequence analyses lead to the conclusion that each of the 1 kb repeats within the duplicated AUD1 copy encodes a putative DNA-binding protein (Piendl *et al.*, 1994) which shares about 90% amino acid identity with the predicted protein encoded by the repeats of the AUD6 element in *S. ambofaciens* (Schneider *et al.*, 1993; Aubert

1249
Fig. 6. Immunological investigations. Substrate hyphae (grown for 5 d) from WT (a), TKIII-10C (b) or TKIII-10T (c) were treated with primary antibodies raised against the 23 kDa protein and secondary fluorescein-labelled antibodies (+) or as control with only secondary antibodies (−). Analyses were done by phase microscopy under visible light (middle) or UV light (left) or under UV light using confocal laser microscopy (right). Scale: (a, right) and (c, right) correspond to the 10 μm bar in (c, right); all other parts correspond to the 10 μm bar in (b, left).
et al., 1993). Further studies are required to understand the molecular events leading to amplification of AUD elements belonging to class I or class II.

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