Altered Lipid Composition in a Non-differentiating Derivative of *Streptomyces hygroscopicus*

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The turimycin-producing *Streptomyces hygroscopicus* strain JA6599/NG60-93 (Tur⁺ Amy⁺) and strain CC1, which is unable to produce antibiotic and aerial mycelium (Tur⁻ Amy⁻), were compared with respect to their mycelial enzyme activities and cellular lipid composition. Changed activities of six enzymes of intermediary metabolism during submerged growth of strain CC1 on chemically defined medium attest to alterations of the life cycle. In addition, strain CC1 contained decreased amounts of 12-methyltetradecanoic acid in relation to 14-methylpentadecanoic acid (isopalmitic acid) and displayed a quantitatively altered phospholipid composition.

INTRODUCTION

Plasmids are known to play an important role in the control of differentiation and secondary metabolism of streptomycetes (Hopwood, 1978). However, at present the biochemical mechanism of plasmid control is unknown (Chater, 1979; Okanishi, 1979). For further study of these problems it should be useful to compare plasmid-carrying strains with their plasmid-free derivatives. During continuous culture the turimycin-producing *Streptomyces hygroscopicus* strain JA6599/NG60-93 (Tur⁺ Amy⁺) segregated non-differentiating clones with high frequency. It was suggested that this segregation might be caused by loss of extrachromosomal DNA (Roth & Noack, 1982). The non-differentiating derivatives were found to be altered with respect to turimycin production (Tur⁻), formation of aerial mycelium (Amy⁻) and resistance to turimycin (Tur^S) compared with their progenitor strain (Tur⁺ Amy⁺ Tur^R) (Roth *et al.*, 1982). In an attempt to ascertain the cellular target of control of differentiation in *S. hygroscopicus*, we have compared the mycelial enzyme activities and lipid composition of the original strain NG60-93 and its non-differentiating derivative CC1 during cultivation on a chemically defined medium.

METHODS

Organisms. Strains NG60-93, NG33-354 and R27-158, mutants of the wild-type strain *Streptomyces hygroscopicus* JA 6599, were obtained from the strain collection of the Zentralinstitut für Mikrobiologie und experimentelle Therapie, Jena. The non-differentiating derivative CC1 was described recently (Roth *et al.*, 1982).

Media. A complex agar medium (CM), AL53 agar (Roth & Noack, 1982) was used for both the propagation of the strains and the cultivation of surface mycelium. Mineral salts medium (Roth & Noack, 1982) containing 10 g glucose l⁻¹, 0.5 g NH₄Cl l⁻¹ and 15 g agar (Difco) l⁻¹ was used for the production of mycelium on minimal agar medium (MM). The medium for submerged culture (SM) contained (g l⁻¹): glucose, 25 (sterilized separately by heat); DL-alanine, 1; DL-aspartic acid, 1; L-glutamic acid, 1; (NH₄)₂SO₄, 1; NaCl, 2.5; KH₂PO₄, 0.5; FeCl₃, 0.005; MgCl₂·6H₂O, 0.04; MnSO₄·2H₂O, 0.02; CaCl₂·6H₂O, 2; CaCO₃, 1; pH 6.2 (prior to sterilization).

Growth conditions. Agar slant cultures were prepared as described previously (Roth & Noack, 1982) except that strain CC1 was incubated for 5 d only. Spores or mycelium from agar slant cultures were spread on the surface of CM and MM medium and incubated for 8 d at 28 °C. All submerged cultivations were carried out in 500 ml glass flasks containing 80 ml medium on rotary shakers (240 rev. min⁻¹, 5 cm stroke). For inocula, spores or mycelium

from agar slant cultures were grown at 27 °C for 48 h and then 3 ml portions were transferred to 80 ml SM medium. These cultures were shaken at 25 °C.

Harvesting of mycelium and disintegration. Mycelium was scraped off the surface of agar plates or collected from submerged cultures by suction filtration. Mycelial extracts were prepared by suspending deep-frozen mycelium (washed and then stored for 1 week at -25 °C) in Tris/HCl buffer (0.1 M, pH 7.2) and subjecting it to sonic treatment for 3 × 30 s (Labsonic 1510, Braun Melsungen, F.R.G.) at 0 °C. The disrupted cells were centrifuged at 0 °C for 15 min (23 000 g) and the supernates were used as crude mycelium extracts for the assays.

Measurements of enzyme activities. Enzyme activities were assayed by standard procedures; all measurements were performed in triplicate. NADP-glutamate dehydrogenase (EC 1.4.1.4) was measured according to Schmidt (1970), NAD-alanine dehydrogenase (EC 1.4.1.1) according to Wiame *et al.* (1962), NAD-malate dehydrogenase (EC 1.1.1.37) according to Bergmeyer (1970), NADP-isocitrate dehydrogenase (EC 1.1.1.42) according to Bernt & Bergmeyer (1970), glutamine synthetase (EC 6.3.1.2) according to Kohlhaw *et al.* (1965), NADP-dependent glucose-6-phosphate dehydrogenase (EC 1.1.1.49) according to Löhr & Waller (1970) and protein by the Lowry method. Turimycin was assayed by means of the standard agar plate diffusion test using *Bacillus subtilis* ATCC 6633 as the test organism.

Lipid extraction and analysis. Lipid material was extracted from the freshly harvested mycelium by treatment with methanol/CHCl₃ (2:1, v/v) for 48 h at room temperature (Kates, 1972). The extraction was repeated for the quantitative estimations. The mycelial content of lipids was estimated gravimetrically. The lipids were obtained from the mycelium extracts by the method of Folch (Kates, 1972). Samples were either subjected to TLC or treated with methanolic HCl (25 g HCl l⁻¹, 48 h) to prepare the methyl esters of fatty acids. TLC was carried out on silica gel sheets (precoated, Merck) using CHCl₃/methanol/water (65:25:4, by vol.) as the solvent for the separation of polar lipids. Authentic samples of phosphatidylethanolamine and cardiolipin (Koch-Light) were run in parallel. For the identification of ornithinolipids, the appropriate zones were scraped off the plates, eluted and hydrolysed for 2 d with 6 M-HCl at 110 °C in sealed glass tubes. The products of hydrolysis were subject to two-dimensional chromatography on precoated cellulose sheets (Merck) according to Arx & Neher (1963). Aminolipids were stained with ninhydrin/collidine reagent while the phospholipids were detected by means of Vaskovski's reagent (Kates, 1972). Separation of neutral and polar lipid fractions was achieved by preparative column chromatography on silica gel 60 (0.065–0.2 mm, Merck) according to Kates (1972).

Gas chromatography. GLC of fatty acid methyl esters was carried out with a gas chromatograph model GCHF-18.3 (VEB Chromatron Berlin, G.D.R.) equipped with a flame-ionization detector. Glass columns (3 m × 3 mm i.d.) were employed, which were filled with 3% (w/w) DEGS on Chromosorb G (80–100 mesh; Serva, Heidelberg, F.R.G.) and operated isothermally at 165 °C. The injection temperature was 220 °C, the detector temperature 200 °C, and nitrogen (35 ml min⁻¹) was the carrier gas. Peaks were identified by comparing their retention times with those of authentic standards of fatty acid methyl esters (Serva). The relative proportions were calculated on the basis of weighed peak areas.

RESULTS

Life cycles of strains NG60-93 and CC1 during submerged growth

During submerged cultivation on SM medium, mutant CC1 differed from the original strain NG60-93 with respect to both its ability to produce the macrolide antibiotic turimycin and its growth pattern (Fig. 1). Thus, strain CC1 displayed prolonged mycelial development and, in contrast to the parent, the mycelium did not settle in the fermentation flask indicating altered morphology of the submerged hyphae (Roth *et al.*, 1982). Differences in the life cycles of strains NG60-93 and CC1 were also shown by changed activities of some enzymes of carbohydrate and amino acid metabolism as well as of the citric acid cycle (Fig. 1). In several independent experiments, strain CC1 displayed higher activities of glutamate and glutamine synthetase and other enzyme activities were also changed.

Lipid composition during submerged and surface culture

The thin-layer chromatograms of aminolipid and phospholipid fractions of submerged mycelium of strains NG60-93 and CC1 are shown in Fig. 2. Phosphatidylethanolamine, two different ornithinolipids (designated ornithinolipids 1 and 2), cardiolipin and a further phospholipid, probably phosphatidylinositol, were the main constituents of mycelial lipids of both strains. As found earlier (Batrakov *et al.*, 1978), addition of phosphate to the SM medium (0.4% KH₂PO₄ added at time 0) led to complete suppression of ornithinolipid formation (U. Gräfe & G. Reinhardt, unpublished observation; Kónova *et al.*, 1978). TLC on silica gel sheets (Fig. 2) gave

Fig. 1. Biochemical characteristics of strains (a) NG60-93 ($\text{Tur}^+ \text{Amy}^+$) and (b) CC1 ($\text{Tur}^- \text{Amy}^-$) during growth on SM medium: biomass ($\times$); pH of the culture medium ($\circ$); turimycin concentration in the medium ($\bullet$); specific enzyme activity [expressed as $\text{nmol min}^{-1} (\text{mg extracted mycelial protein})^{-1}$] - $0.01 \times$ NAD-malate dehydrogenase ($\square$), $0.1 \times$ NADP-isocitrate dehydrogenase ($\blacksquare$), NADP-glutamate dehydrogenase ($\triangle$), $0.1 \times$ NAD-alanine dehydrogenase ($\blacktriangle$), glutamine synthetase ($\blacktriangledown$), $0.5 \times$ NADP-glucose-6-phosphate dehydrogenase (∇).

the same R_F values for cardiolipin and ornithinolipid 1. However, on silica gel H plates (Merck) both spots could be well separated (Kates, 1972). The lipids of both strains showed no striking difference in their qualitative composition although the ratios of their components were altered. Thus, during the growth of strain CC1 there was a reduction in the amount of phosphatidylethanolamine while the amount of ornithinolipid 2 increased. Similar lipid compositions were found in mycelium harvested from agar plate cultures on CM and MM medium, except that ornithinolipid 2 was not present. Differences were also detected in the total lipid content of submerged cultures grown for 72 h in SM medium. In this case strain CC1 contained about twice as much lipid material (2.1%, w/w, of mycelial dry weight) as did the parent strain NG60-93 (0.8%, w/w).

Mycelial fatty acids

The results of the GLC analysis of methyl esters of fatty acids extracted from mycelium grown on the surface of CM and MM medium are presented in Table 1. Under these conditions, strain CC1 contained markedly higher concentrations of alkenic fatty acids (18:1; 18:2) and the amount of isopalmitic acid (i16:0) relative to 12-methyltetradecanoic acid (a15:0) was also increased. Differences in the ratio of a15:0 to i16:0 were also found during submerged culture in SM medium (Table 2). These investigations were extended to three other strains of *S. hygroscopicus* JA6599 yielding either high (NG33-354, R27-158) or low (wild-type strain) amounts of turimycin. All of them were capable of forming aerial mycelium (Amy^+). These $\text{Tur}^+ \text{Amy}^+$ strains contained almost equal portions of a15:0 and i16:0, whereas CC1 possessed a drastically

Table 1. Relative proportions (%) of fatty acids from mycelia from agar plate cultures of strains NG60-93 and CC1 grown for 8 d on CM and MM medium

Medium	Strain	i14:0	14:0	a15:0	i16:0	16:0	16:1/ a17:0	18:0	18:1	18:2	UI	a15:0/ i16:0
MM	NG60-93	11.2	—	34	33.6	6.6	12.3	—	—	—	2.0	1.0
	CC1	13.8	2.4	14.6	29	14.6	7.2	1.6	1.6	4	9.7	0.5
CM	NG60-93	14.5	—	25	43	9.3	6	—	—	—	2	0.6
	CC1	10.5	3.7	10.1	43.5	5.6	3	1.5	9	5.2	7.5	0.23

Abbreviations for fatty acids: 14:0, tetradecanoic acid; i14:0, 12-methyltridecanoic acid; a15:0, 12-methyl-tetradecanoic acid; i16:0, 14-methylpentadecanoic acid (isopalmitic acid); 16:0, palmitic acid; 16:1/a17:0, non-separated peak of both hexadecenic and 14-methylhexadecanoic acids; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; UI, unidentified peak with longer retention time than 18:2.

Fig. 2. TLC separation of mycelial lipids of strains NG60-93 and CC1 grown on SM medium: (a) aminolipids (ninhydrin stain); (b) phospholipids (Vaskovski's stain). Each track corresponds to 0.1 mg of lipid sample.

reduced amount of the $\alpha 15:0$. Only minor differences were observed for the other fatty acids present. An additional unidentified fatty acid (with a longer retention time than $18:2$), present in all strains, was believed to be a substituent of the ornithinolipids which are known to contain unusual hydroxy fatty acids (Batrakov & Bergelson, 1978). In other experiments, the total lipids of 72 h cultures of strains NG60-93 and CC1 were separated into non-polar (neutral) lipids (25% of the total lipids) and polar lipids (phospholipids and ornithinolipids, 75%) by column chroma-

Table 2. Relative proportions (%) of fatty acids from mycelia of strains JA6599 (wild-type), NG33-354, R27-158, NG60-93 and CC1 during growth on SM medium

Strain	Time (h)	14:0	i14:0	a15:0	i16:0	16:0	16:1/ a17:0	18:0	18:1	18:2	UI	a15:0 i16:0
NG60-93	24	5	2	25.5	20.5	9	14	2	2	10	10	1.24
	48	2.8	tr.	23.6	21.9	9.8	9.8	1.2	6.9	10.9	12.7	1.07
	72	1.7	tr.	12.1	10	6.9	4.5	2	10	14	38	1.21
CC1	24	4.3	1.4	9.5	29.4	9.2	14.2	1.9	6.7	12	12	0.34
	48	2	tr.	8.1	25.2	12.3	20	tr.	4.7	4.6	23.2	0.32
	72	1.7	tr.	6.7	29.6	4.8	11.6	tr.	6.2	6.4	33.2	0.22
NG33-354	24	5.9	1.9	26.9	21.7	12.3	8.6	4.2	6.2	2.4	10	1.23
	48	7.1	tr.	38.4	25.6	12.1	10.6	tr.	tr.	1	5	1.50
	72	4.9	tr.	33	25.8	9.9	13.2	tr.	tr.	1	12	1.28
R27-158	24	8.1	0.5	30	20	20.6	8.9	1.2	1.8	3.1	6	1.50
	48	2.6	tr.	13.6	15.2	14.7	9.2	3.4	9.2	16.5	16.5	0.90
	72	4.9	tr.	33.6	23.4	7.6	12.7	1.4	4.3	4.3	7.3	1.43
JA6599	24	4.7	tr.	18	16.2	29.8	5.1	4.4	5.8	5.1	11.1	1.11
	48	6.7	tr.	27.9	25.1	15.7	9.9	2.4	1.9	0.6	10	1.11
	72	4	tr.	22.4	32	12.5	16.2	tr.	2.9	2.7	7.6	0.70

Abbreviations for fatty acids as in Table 1; tr., trace.

tography on silica gel. The ratio of a15:0 to i16:0 was particularly low in the polar lipids fraction of strain CC1 (a15:0/i16:0 was 0.5 for the neutral lipids and 0.15 for the polar lipids).

DISCUSSION

Our results demonstrate that the lipid composition of the Tur⁻ Amy⁻ derivative CC1 differs from that of its parent strain NG60-93. This raises the possibility that concomitant changes of membrane-associated functions may be responsible for alteration of the life cycle of these organisms. Alterations of lipid composition have been observed in mutants of hygromycin-producing *Streptomyces hygroscopicus* obtained by treatment with acridine orange or after spontaneous degeneration (Pronina *et al.*, 1980). Further, a great number of sporulation mutants of *Bacillus* species (Schaffer *et al.*, 1972) are known to have an altered lipid composition. Our results also suggest that changes of membrane composition correspond to the inability of streptomyces mycelia to differentiate (Kalakoutski & Agre, 1977; Ensign, 1978). On the basis of the supposed plasmid involvement in the control of differentiation processes in *S. hygroscopicus* (Roth & Noack, 1982), it appears likely that this plasmid might be linked with the regulation of membrane composition. The altered ratio of a15:0 to i16:0 fatty acids in the derivative CC1 compared with the original strain is consistent with the view that there are alterations in the production of the precursors 2-methylbutyryl-coenzyme A and isobutyryl-coenzyme A (Machtinger & Fox, 1973; Murray & Magee, 1972). Both precursors are known to be produced via the degradation of methyl-branched amino acids. The findings support the view that the observed alterations of fatty acid composition during growth on chemically defined medium can be due to peculiar changes in either the formation of valine and isoleucine or in the structure and function of the common ketoacid dehydrogenase complex which is linked with their catabolism (Willecke & Pardee, 1971).

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