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Nitrogen regulation of fatty acids and avermectins biosynthesis in *Streptomyces avermitilis*

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1. SUMMARY

Fatty acid composition was analysed in the producer of avermectins, *Streptomyces avermitilis* C-18 grown in chemically defined medium with different nitrogen sources. Significant differences in nitrogen regulation of fatty acid biosynthesis were found in this strain in comparison with other streptomycetes studied so far. This finding could be explained at the level of regulation of branched-chain amino acid metabolism.

2. INTRODUCTION

Avermectins are 16-membered antiparasitic macrolides produced by *Streptomyces avermitilis* [1–3]. The lactone ring of the most common types (B1a, B2a, A1a, A2a) is formed from one methylbutyrate, seven acetate and five propionate units [4]. A disaccharide α-L-oleandrosyl-α-L-oleandrosid is attached to the C-13 of the lactone ring.

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Overproduction of lipids was reported in *S. avermitilis* [5]. Cellular lipids contained mostly triglycerides and phospholipids with high content of terminally branched fatty acids. A quantitative and temporal correlation was found between the production of avermectins and triglycerides in *S. avermitilis* grown in chemically defined media [6]. Those two groups of metabolites are supposed to compete for common precursors.

Methylbutyrate as starting unit of anteiso fatty acids [7] or avermectins [8] could originate from de novo synthesis of isoleucine carbon chain [6,9] or directly from isoleucine catabolism through transamination or deamination and oxidative decarboxylation [10]. A mutant strain lacking branched-chain 2-oxo acid dehydrogenase activity was blocked in avermectin production. This was restored by supplementation with methylbutyrate or isobutyrate [10]. Other carboxylic acids fed during cultivation were used as alternative starting units of avermectins yielding novel avermectins [11,12].

Biosynthesis of avermectins is significantly influenced by nitrogen sources used in cultivation medium. Optimized chemically defined medium contains ammonium sulfate. Higher concentra-

tions deminish the yield and isoleucine as the only nitrogen source completely inhibits the production of avermectins [13]. The interference of nitrogen metabolism with the synthesis of precursors of avermectins is proposed as an important mechanism of depression of avermectin biosynthesis by high ammonium concentrations [14].

Nitrogen regulation of fatty acid composition in *S. avermitilis* is described in this paper. The relations among branched-chain amino acid metabolism and fatty acid and secondary metabolite biosynthesis in streptomycetes are discussed.

3. MATERIALS AND METHODS

3.1. Organism, media and growth condition

A mutant strain of *S. avermitilis* C-18 derived from strain ATCC 31267 was used [13]. Preparation of inoculum and cultivation conditions in chemically defined medium were described earlier [6].

3.2. Analytical methods

Determination of avermectins (TLC) and fatty acids (GC-MS) has already been described [6]. Dehydrogenase and transaminase of branched-chain amino acids were assayed spectrophotometrically according to [15] and [16], respectively. Proteins were determined as in [17]. *S. aureofaciens* was used as a positive control for measurement of branched-chain amino acid dehydrogenase activity.

4. RESULTS AND DISCUSSION

Analysis of fatty acids of *S. avermitilis* grown in defined medium containing various nitrogen sources of supplements is given in Tables 1 and 2. While the yield of both avermectins and lipids is influenced by ammonium content in medium [6], the simple correlation between avermectin production or avermectin B/A ratio and fatty acid composition was not found (Tables 1, 3). All the identified fatty acids were in the range C_{13}–C_{18}. Terminally branched fatty acids amounted to 60–70% in chemically defined medium with different amounts of ammonium salts. No significant changes were found in fatty acid composition when comparing mycelium growing in medium supplemented with 0.05% or 0.5% ammonium sulfate (Table 1, samples 1, 2). Table 2 shows the composition of fatty acids during cultivation. At the beginning of growth, there was a small shift in the fatty acid composition, similar as described in *S. cyaneus* [18], but the fatty acid profiles remained relatively constant during the rest of cultivation and similar in media with both low and high amount of ammonium sulfate.

The addition of 2-methylbutyrate to the basal medium with ammonium as a nitrogen source led to the increase of anteiso-fatty acids and decrease of straight-chain fatty acids (Table 1, samples 4, 5). The amount of iso-even and -odd fatty acids was not changed. This is in agreement with the pathway isoleucine → methylbutyrate → anteiso-fatty acids, suggesting there is no cross-talk in biosynthesis of iso- and anteiso-fatty acids using 2-methylbutyrate as a precursor. Isoleucine used as the only nitrogen source changed the composition of fatty acids significantly: anteiso-fatty acids were prevailing (90%) while iso-even and -odd fatty acids formed only 4% of total (Table 1, sample 3). This finding could be interpreted in this way: isoleucine as the only nitrogen source inhibited metabolism of branched amino acids serving as building units for iso-branched fatty acids. This is in agreement with previous findings that isoleucine inhibites the growth of mycelium, glucose consumption and biosynthesis of avermectins [13,19]. Though the mechanism of isoleucine action remains obscure, the understanding of this effect could also help to elucidate the selective production of specific avermectins of group 'a' with a starting unit derived from isoleucine [20].

Nitrogen depression of antibiotic biosynthesis is well known is streptomycetes [21]. However, the relations between nitrogen metabolism and the biosynthesis of lipids and antibiotics was studied in a limited number of species. In regulatory mutants of *S. cinnamonensis* resistant to amino acid analogues, the effect of change in biosynthesis of endogenous precursors on the composition of fatty acids was described [22,23]. The overpro-

Table 1

Composition of the main fatty acids in *S. avermitilis* after 10 days of cultivation in a chemically defined medium with different nitrogen sources or supplements. Each sample is the average from 3 to 5 flasks

FA [a]	Sample number [b]				
	1	2	3	4	5
i-13:0	0.1	0.1	0.0	0.0	0.0
ai-13:0	0.2	0.3	2.5	0.6	0.5
13:0	0.0	0.0	0.0	0.0	0.0
i-14:0	4.0	3.1	0.1	2.4	2.5
14:0	1.5	1.6	0.4	0.5	0.5
i-15:0	5.8	5.7	0.0	4.4	4.4
ai-15:0	21.3	21.8	62.9	34.1	32.4
15:1	0.2	0.2	0.0	0.0	0.0
15:0	5.4	3.5	0.7	1.1	1.4
i-16:0	19.1	16.8	0.5	20.4	20.9
16:1	7.2	6.6	0.9	4.4	4.3
16:0	20.3	21.8	3.5	5.4	7.8
i-17:0	1.7	2.6	3.5	1.2	1.5
ai-17:0	10.1	11.4	24.8	23.0	21.4
17:1	1.7	2.0	0.1	1.0	1.2
17:0	1.4	1.2	0.1	0.0	0.0
18:1	0.0	0.9	0.0	1.0	0.9
18:0	0.0	0.4	0.0	0.5	0.3
i_e	23.1	19.9	0.6	22.8	23.4
i_o	7.6	8.4	3.5	5.6	5.9
a_i	31.6	33.5	90.2	57.7	54.3
n_e	29.0	31.3	4.8	11.6	13.8
n_o	8.7	6.9	0.9	2.3	2.6

[a] Relative proportions of the individual fatty acids in the sample (mass %): i, isoacid; ai, anteisoacid; e/o, even/odd. The first number indicates the number of carbon atoms. The second the number of unsaturated bonds in the molecule.

[b] Synthetic medium with ammonium sulfate 0.05% (1), 0.5% (2), 0.2% (4, 5) or isoleucine 0.2% (3) as the only nitrogen source, respectively. 2-Methylbutyrate (1) mg/ml, final concentration) was added in 5 consecutive portions (day 5–9; column 4) or in one portion (day 4; column 5).

duction of valine is supposed to be the reason for both increased yield of monensin A and higher content of iso-even fatty acids, where valine serves as a precursor for isobutyrate or (after isomerization) butyrate units. The effect of exogenous substances on fatty acid composition and biosynthesis of 16-membered macrolide tylosin was studied in *S. fradiae* [25,26]. High ammonium concentration inhibits the production of tylosin and decreases the quantity of branched-chain fatty acids, while total lipids remain constant. The mechanism of ammonium action is based on the inhibition of precursor formation from amino acids and intermediary metabolism. At the enzyme level, repression and inhibition of valine dehydrogenase (EC 1.4.1.8) – the first enzyme in valine catabolism – by ammonium have been described.

On the contrary, in *S. avermitilis* the yield of lipids and avermectins was influenced by ammonium while the composition of fatty acids remained constant. The total synthesis of oligoketides in *S. avermitilis* seemed to be regulated at the level of global metabolic network. However, previous research showed that enzyme patterns in branched-chain amino acid metabolism significantly influences the biosynthesis of oligoketides [10,19,26]. Dehydrogenase of branched-chain amino acids was not found in *S. avermitilis* under any cultivation condition [19]. Transaminase B (EC 2.6.1.42) seemed to be the only enzyme

Table 2

Composition of the main fatty acids in *S. avermitilis* during cultivation in a chemically defined medium. Each sample is the average from 3 to 5 flasks

FA[a]	days									
	2[b]	4[b]	6[b]	8[b]	10[b]	2[c]	4[c]	6[c]	8[c]	10[c]
i-13:0	0.1	0.1	0.1	0.2	0.1	0.0	0.0	0.2	0.2	0.1
ai-13:0	0.1	0.3	0.2	0.3	0.2	0.0	0.0	0.3	0.3	0.3
13:0	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.0
i-14:0	4.3	4.7	4.8	4.5	4.0	2.0	3.1	3.4	2.9	3.1
14:0	1.0	1.6	1.8	1.6	1.5	1.0	1.3	1.8	1.5	1.6
i-15:0	5.8	6.2	5.9	7.1	5.8	4.3	5.7	6.0	5.2	5.7
ai-15:0	20.7	22.0	20.3	21.2	21.3	18.8	25.7	21.1	20.2	21.8
15:1	0.0	0.1	0.2	0.2	0.2	0.0	0.0	0.1	0.0	0.2
15:0	2.4	4.3	5.1	5.4	5.4	2.0	2.8	4.0	3.0	3.5
i-16:0	20.8	19.7	18.3	18.7	19.1	13.9	19.7	18.0	17.0	16.8
16:1	12.0	7.3	8.8	7.4	7.2	17.5	5.3	5.4	10.6	6.6
16:0	13.0	19.0	20.7	18.3	20.3	13.6	15.5	21.7	18.9	21.8
17:0	2.7	1.7	1.6	2.0	1.7	4.4	4.7	2.4	2.8	2.6
ai-17:0	9.6	9.5	8.7	9.5	10.1	9.7	13.4	11.6	11.5	11.4
17:1	1.6	1.6	1.5	1.9	1.7	0.0	2.0	2.2	1.9	2.0
17:0	0.6	0.9	1.1	1.2	1.4	1.2	0.8	1.2	1.0	1.2
18:1	4.7	0.7	0.7	0.3	0.0	10.1	0.0	0.3	2.6	0.9
18:0	0.5	0.2	0.1	0.1	0.0	1.5	0.0	0.2	0.4	0.4
i_e	25.1	24.4	23.1	23.2	23.1	15.9	22.8	21.4	19.9	19.9
i_o	8.6	8.0	7.6	9.3	7.6	8.7	10.4	8.6	8.2	8.4
a_i	30.4	31.8	29.2	31.0	31.6	28.5	39.1	33.0	32.0	33.5
n_e	31.2	28.8	32.1	27.7	29.0	43.7	22.1	29.4	34.0	31.3
n_o	4.7	7.0	8.0	8.8	8.7	3.2	5.6	7.6	5.9	6.9

[a] Relative proportions of the individual fatty acids in the sample (mass%): i, isoacid; ai, anteisoacid; e/o, even/odd. The first number indicates the number of carbon atoms. The second the number of unsaturated bonds in the molecule.
[b] Initial concentration of 0.05% ammonium sulfate was used.
[c] Initial concentration of 0.5% ammonium sulfate was used.

catalysing branched-chain amino acid catabolism in this strain (Table 4). The possibility that presence and expression of particular isoenzymes of

Table 3

Production of avermectins in *S. avermitilis* after 10 days of production on in chemically defined media with different nitrogen sources. Each sample is the average from 3 flasks

Medium[a]	1	2	3	4
AVM[b]	100	69	44	0
B/A[c]	1.04	1.01	0.16	–

[a] Synthetic medium with 0.2% (1), 0.05% (2), 0.5% (3) ammonium sulfate or 0.2% isoleucine (4) as the only nitrogen source, respectively.
[b] Relative production of avermectins (%).
[c] Avermectins B/ avermectins A ratio.

Table 4

Activity of enzymes involved in catabolism of branched-chain amino acids in *S. avermitilis*

Medium[a]		1	2
BCAADH[b]		ND	ND
BCAAT[c]	leu	0.19	0.21
	ile	0.10	0.05
	le/ile ratio	1.9	4.2

[a] Synthetic medium with 0.2% ammonium sulfate (1) or 0.2% isoleucine (2) as the only nitrogen source, respectively. Mycelium was harvested after 1 (1) or 2 days (2) of cultivation.
[b] Branched-chain amino acid dehydrogenase.
[c] Branched-chain amino acid transaminase (transaminase B); kat $\times 10^{-6}$ (g protein)$^{-1}$. ND not detected.

transaminase B are differentially regulated, suggested by a changed ratio of transaminase B activity with leucine and isoleucine as substrates using samples from various media (Table 4), could not be excluded.

Results presented in this paper suggest that nitrogen regulation of avermectins and fatty acid biosynthesis in *S. avermitilis* could proceed by a novel mechanism, different from nitrogen depression of tylosin biosynthesis. The understanding of the regulatory mechanism could have serious implications for the strategy of increasing the avermectin yield.

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