Resistance of *Streptomyces cinnamomensis* to butyrate and isobutyrate: production and properties of a new anti-isobutyrate (AIB) factor

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Butyrate and isobutyrate (after isomerization to *n*-butyrate) are specific precursors for the biosynthesis of monensin A in *Streptomyces cinnamomensis*. High concentrations of both butyrate and isobutyrate (greater than 20 and 10 mM, respectively) were toxic to *S. cinnamomensis* plated on solid medium. Spontaneous mutants resistant to these substances were isolated. These new strains produced monensins at even higher concentrations of butyrate or isobutyrate, with an increased yield of monensin A. *S. cinnamomensis* produced an anti-isobutyrate (AIB) factor, which was originally found to be excreted by some isobutyrate-resistant strains growing on solid medium containing isobutyrate. On plates, the AIB factor efficiently counteracted toxic concentrations not only of isobutyrate, but also of acetate, propionate, butyrate, 2-methylbutyrate, valerate and isovalerate against *S. cinnamomensis* as well as other *Streptomyces* species. Although the AIB factor enabled normal growth, sporulation and monensin production on plates, it did not have positive effects on submerged cultures of *S. cinnamomensis* with isobutyrate. The partial purification of the AIB factor was achieved. The role of the AIB factor during spore germination on solid medium containing isobutyrate or its homologues is discussed.

Introduction

Streptomyces cinnamomensis is the producer of the polyether antibiotics monensins (Haney & Hoehn, 1968). Monensins A and B, produced in approximately equal amounts, are the major secondary metabolites of standard *S. cinnamomensis* strains. Monensin A exhibits higher biological activity against *Bacillus subtilis* than monensin B (Gorman *et al.*, 1968) and it is used as a coccidiostatic agent. From the biogenetic point of view, monensins are classified as oligoketides. The basic skeleton of monensin A is assembled by the sequential coupling of precursors derived from one butyrate, seven propionate and five acetate building blocks (Day *et al.*, 1973). In monensin B, the butyrate is substituted by an eighth propionate unit and the molecule thus contains a methyl instead of an ethyl group (Fig. 1).

In previous studies, we showed that the butyrate unit forming the ethyl group of monensin A is derived from valine and its metabolite isobutyrate. This implied the existence of a new metabolic pathway including the isomerization of isobutyrate to *n*-butyrate (Pospíšil *et al.*, 1983).

The monensin A/B ratio can be influenced by the extracellular level of the corresponding precursors, e.g. by amino acids added to the cultivation medium (Pospíšil *et al.*, 1982) or intracellularly, i.e. by the overproduction of valine in regulatory mutants (Pospíšil *et al.*, 1984). Feeding with direct precursors was also partially effective. Using propionate, the proportion of monensin B increased, whereas butyrate and isobutyrate favoured monensin A production (Pospíšil *et al.*, 1985). However, the effect of these substances was low due to their adverse effects on the producer.

The aim of the present work was to obtain strains of *S. cinnamomensis* resistant to higher concentrations of

Monensin A: R = CH₂CH₃

Monensin B: R = CH₃

Fig. 1. Structure of monensins.

Abbreviations: AIB, anti-isobutyrate; BR, butyrate-resistant; IBR, isobutyrate-resistant.

butyrate and isobutyrate and to compare their properties. A preliminary report of the findings was given at the IUMS Congress of Bacteriology and Mycology, Osaka, September 16–22, 1990.

Methods

Organisms. *S. cinnamomensis* C-100-5 was obtained by a multistep selection from strain ATCC 15413 (K. Klánová, personal communication). *Streptomyces coelicolor* 165, *Streptomyces griseus* IPB 101, *Streptomyces spectabilis* NRRL 2494 and *Streptomyces virginiae* ATCC 13161 from the laboratory collection were used for testing the AIB factor.

Media and cultivation. All strains were maintained and cultivated during experiments on yeast extract/malt extract agar (Hesseltine *et al.*, 1954) at 28 °C. Submerged cultivation of *S. cinnamomensis* was carried out in 500 ml flat-bottomed flasks containing 50 ml of a medium containing (g l⁻¹): glucose, 25 (seed medium) or 50 (production medium); soybean flour, 15; CaCO₃, 3; FeSO₄, 0.3; MnCl₂·4H₂O, 0.03. After 30 h cultivation, the seed culture was used as an inoculum (2%, v/v) for the production medium. Cultivations were performed on an orbital shaker (162 r.p.m.) at 34 °C for 6.5 d. Butyrate and isobutyrate were added to the production medium as sodium salts in the form of sterile aqueous solutions before inoculation. All fermentations and monensin assays were done in triplicate.

Isolation of mutants resistant to butyrate and isobutyrate. Agar medium containing butyrate or isobutyrate (10–50 mM) was inoculated with a dense spore suspension of *S. cinnamomensis* C-100-5. The plates were incubated in a humid chamber for several weeks until resistant colonies appeared. Both butyrate-resistant (BR) and isobutyrate-resistant (IBR) strains were obtained under the same conditions. The BR strains were directly inoculated on agar slants. To assure homogeneity of the IBR strains, the cells were spread on agar medium with the corresponding concentration of isobutyrate and afterwards routinely reinoculated.

Assay of production of monensins. The total production of monensins after submerged cultivation of *S. cinnamomensis* was determined as described previously (Pospíšil *et al.*, 1986). The monensin A/B ratio was ascertained densitometrically (Pospíšil & Zima, 1987). Monensins formed during surface cultivation on agar in Petri dishes (9 cm diameter, 25 ml) were extracted three times with 30 ml methanol. The agar with the mycelium was disintegrated with sand in a mortar. Crude extracts were diluted with water and monensins were re-extracted with chloroform. The chloroform phases were evaporated *in vacuo*. The dried extracts were resuspended in 0.1 ml chloroform for analysis by thin-layer chromatography (Pospíšil *et al.*, 1986).

Partial purification of the AIB factor. After the submerged cultivation of IBR strains producing the AIB factor, the culture broth was filtered and the filtrate was adjusted to pH 1.5 with 2 M-HCl and left overnight in the cold. The precipitate was centrifuged (6000 g, 15 min) and washed several times with 10 mM-HCl. The AIB factor was released from the precipitate with 2 M-NH₄OH. The extraction was repeated at least five times with excess NH₄OH. The combined extracts, after evaporation of ammonia, were fractionated by elution from an LH-20 Sephadex column with water. The presence of the AIB factor in the fractions was detected by bioassay. A sample (0.1 ml) of the tested fraction was pipetted into a well in agar medium containing 20 mM-isobutyrate and inoculated with a spore suspension of *S. coelicolor* 165. The growth of the test organism in the diffusion zone around the well provided evidence for the presence of the AIB factor. For biological experiments, the solution of the AIB factor was sterilized by membrane

filtration. One unit of activity (U) was defined as the minimal amount of the AIB factor per 0.1 ml of the tested solution required to induce growth of *S. coelicolor* 165 under the assay conditions. *S. coelicolor* was used because of the production of blue substances which improve detection ability.

Assay of the activity of AIB factor in surface cultivation. Agar medium containing a sodium salt of acetic, propionic, butyric, isobutyric, 2-methylbutyric, valeric or isovaleric acid (each 20 mM) was inoculated with the streptomycete spore suspensions. The solution of the AIB factor (0.1 ml, 2 U) was added to wells in the centre of Petri dishes. Its effect was indicated by the growth and sporulation after incubation for 5–7 d.

Assay of the activity of the AIB factor in submerged cultivation. To 50 ml liquid medium, isobutyrate (20 mM) and the solution of the AIB factor (1, 5, 10 or 20 U) were added. This medium, together with the control, was inoculated with *S. cinnamomensis* C-100-5 spores (one-step cultivation) or with a 30 h inoculum. After 6.5 d cultivation, the production of monensins was assayed.

Chemicals. 2-Methylbutyric acid, valeric acid and isovaleric acid were purchased from Aldrich; the others were from Lachema. These acids were neutralized to pH 8 with NaOH and 1 M stock solutions were prepared.

Results

Properties of resistant strains

Different levels of sensitivity of the standard strain *S. cinnamomensis* C-100-5 to butyrate and isobutyrate were observed during the isolation of resistant strains. The lowest butyrate concentration used (10 mM) made rapid growth and sporulation of the whole cell population possible. In contrast, on agar containing the same concentration of isobutyrate, individual colonies appeared only after 7 d cultivation (Table 1). Spores of *S. cinnamomensis* C-100-5 were more sensitive to isobutyrate. Primary colonies of isobutyrate-resistant strains were gradually surrounded by a growing population of the standard strain as a consequence of the production of an unknown substance eliminating the toxic effect of isobutyrate, while colonies of butyrate-resistant strains were not. This new substance was named anti-isobutyrate (AIB) factor. Out of a total of 19 colonies appearing on agar with 30 mM-isobutyrate, 16 dwarf colonies did not excrete the AIB factor.

The effect of butyrate and isobutyrate on monensin production was studied in submerged cultures with all resistant strains obtained. The results were compared with the response of the parent strain C-100-5. A complex influence of both specific monensin A precursors was reflected in the relative yield of monensin A. Table 2 shows selected BR strains that gave a higher relative yield of monensin A in the first step of strain evaluation. The data in Table 2 also demonstrate that monensin production in the resistant strains under

Table 1. *Origin of spontaneous mutants of S. cinnamomensis C-100-5 resistant to butyrate and isobutyrate*

Concn (mM)	Butyrate			Isobutyrate		
	Time of appearance of colonies (d)	No. of colonies	Strain	Time of appearance of colonies (d)	No. of colonies	Strain
10	2	∞	—	7	9	10-IBR
20	28	2	20-BR	14	2	20-IBR
30	35	23	30-BR	35	19	30-IBR
40	45	7	40-BR	—	0	—
50	—	0	—	—	0	—

Table 2. *Effect of butyrate on the production of monensins in submerged cultures of S. cinnamomensis C-100-5 and its derived resistant mutants*

Strain	Concn of butyrate (mM)	Production of monensins ($\mu\text{g ml}^{-1}$)	Proportion of monensin A (%)	Relative yield of monensin A* (%)
C-100-5	0	387	49	49
	30	77	68	13.5
30-BR-4	0	480	46	46
	30	435	65	58.9
30-BR-5	0	475	50	50
	30	464	72	70.3
40-BR-5	0	465	60	60
	30	136	80	23.4

* Relative yield of monensin A = (production of monensins/production of monensins in cultures without butyrate) $\times$ proportion of monensin A.

Table 3. *Effect of isobutyrate on the production of monensins in submerged cultures of S. cinnamomensis C-100-5 and its derived resistant mutant 30-IBR-3*

Strain	Concn of isobutyrate (mM)	Production of monensins ($\mu\text{g ml}^{-1}$)	Proportion of monensin A (%)	Relative yield of monensin A* (%)
C-100-5	0	256	45	45
	10	91	55	19.6
	20	8	69	2.2
30-IBR-3	0	365	43	43
	10	285	55	42.9
	30	229	70	43.9

* Relative yield of monensin A = (production of monensins/production of monensins in cultures without isobutyrate) $\times$ proportion of monensin A.

Fig. 2. Production of the AIB factor by *S. cinnamomensis* C-100-5 under unrestricted conditions. *S. cinnamomensis* was grown on standard agar medium for 7 d. A strip was cut from this agar and laid on agar medium containing an inhibitory concentration of isobutyrate (40 mM) and inoculated with *S. coelicolor* 165 spores. Diffusion of the AIB factor from the agar strip permitted growth of *S. coelicolor* 165. Photographed after 10 d cultivation.

standard conditions, i.e. without butyrate, was higher as compared with the parent standard strain.

Out of the 30 IBR strains tested, only 30-IBR-3 (simultaneously producing the AIB factor) exhibited higher production in submerged culture than the standard strain C-100-5 (Table 3). The production activity of the remaining IBR strains was lower than or, at most, the same as the production of the parent strain C-100-5. All IBR strains secreting the AIB factor during surface growth also produced this substance in submerged cultures, irrespective of the presence of isobutyrate in the medium. Strain 10-IBR-9, a mutant blocked in monensin biosynthesis, produced the AIB factor as well.

As may be noted in Tables 2 and 3, butyrate and isobutyrate favoured the biosynthesis of monensin A. Both substances showed no or diminished inhibitory effects on the total antibiotic production in BR and IBR strains as compared with the parent strain C-100-5.

Owing to the well-known genetic instability of actinomycetes (Cullum *et al.*, 1988), the selected resistant strains were repeatedly tested in submerged culture after reinoculation into another slant culture. However, monensin production in all strains dropped to the original value of the standard strain C-100-5. AIB factor production was a stable marker.

Table 4. Effect of the AIB factor on the production of monensins in submerged cultivation of *S. cinnamomensis* C-100-5 in the presence of isobutyrate

Concn of isobutyrate (mM)	Concn of AIB factor (10 × U ml ⁻¹)	Production of monensins (µg ml ⁻¹)	
		One-step culture	Two-step culture
0	0	464	688
20	0	288	160
20	0.2	200	160
20	1.0	100	128
20	2.0	88	54
20	4.0	64	32

In control experiments, the excretion of the AIB factor by wild-type *S. cinnamomensis* C-100-5 was examined under conditions of standard surface and submerged culture without isobutyrate. The AIB factor was readily detected in agar (Fig. 2) but only traces were found in the tenfold concentrated broth after submerged culture. The direct estimation of AIB factor production in the fermentation broth was impossible due to the presence of undefined substances which adversely influenced the bioassay.

Properties of the AIB factor

Preliminary experiments proved that the AIB factor was able to thoroughly suppress the effect of toxic concentrations of isobutyrate and facilitated normal growth and sporulation on agar. It was therefore desirable to find out whether the AIB factor acts on strain C-100-5 during submerged growth and makes normal levels of monensin production in the presence of isobutyrate possible. Partially purified AIB factor was added to submerged cultures of strain C-100-5 at the beginning of the fermentation. Amounts of 1, 5, 10 or 20 U of the AIB factor in the form of a sterile solution were added to the liquid medium with 20 mM-isobutyrate. Small doses of the AIB factor (1 U) had either no effect or a negative effect (Table 4), while higher concentrations of the factor significantly inhibited the production of monensins. The experiments were performed in both two-step and one-step cultures to ensure the presence of the AIB factor during spore germination. The response was negative in both cases.

The effectiveness of the AIB factor was also determined on solid medium. Because earlier studies (unpublished results) had shown that, in addition to butyrate

Fig. 3. Effect of the AIB factor on *S. cinnamomensis* C-100-5 inoculated on agar medium containing an inhibitory concentration (20 mM) of the following substances: A, acetate; P, propionate; B, butyrate; IB, isobutyrate; MB, 2-methylbutyrate; V, valerate; IV, isovalerate. C is the control. The solution of the AIB factor (2 U) was added to the wells in the centre of the Petri dishes (lower rows in each photograph).

and isobutyrate, salts of other low molecular mass aliphatic monocarboxylic acids exhibited a toxic effect on *S. cinnamomensis*, acetate, propionate, 2-methylbutyrate, valerate, and isovalerate were included in the experiment. The effect at the toxic concentration of 20 mM was suppressed in each case by the AIB factor (Fig. 3). TLC analysis of agar extracts showed that the amount of monensins produced was similar to that in the control culture (data not shown).

To see whether the AIB factor had a broader effect, not confined to *S. cinnamomensis*, its influence on several selected streptomycete species was studied. As shown in Fig. 4, the AIB factor, in the presence of a toxic concentration of isobutyrate, restored growth, sporulation and pigment formation in all species tested, but it was not effective in some species against salts of some of the other low molecular mass aliphatic monocarboxylic acids used (Table 5).

The pH of the AIB factor solutions used was neutral and therefore it could not influence spore germination.

Fig. 4. Effect of the AIB factor on different species of streptomycetes inoculated on agar medium containing an inhibitory concentration (20 mM) of isobutyrate. 1, *S. coelicolor* 165; 2, *S. griseus* IPB 101; 3, *S. spectabilis* NRRL 2494; 4, *S. virginiae* ATCC 13161. The solution of the AIB factor (2 U) was added to the wells in the centre of the Petri dishes (lower row).

Table 5. Effect of the AIB factor on different species of streptomycetes inoculated on agar medium containing an inhibitory concentration (20 mM) of various monocarboxylic acids

Strain	A	P	B	IB	MB	V	IV
<i>S. cinnamonensis</i> C-100-5	+	+	+	+	+	+	+
<i>S. coelicolor</i> 165	+	0	+	+	+	+	+
<i>S. griseus</i> IPB 101	+	+	+	+	+	+	+
<i>S. spectabilis</i> NRRL 2494	+	+	+	+	0	0	0
<i>S. virginiae</i> ATCC 13161	0	0	0	+	0	0	0

Abbreviations and symbols: A, acetate; P, propionate; B, butyrate; IB, isobutyrate; MB, 2-methylbutyrate; V, valerate; IV, isovalerate; +, AIB factor effective in restoring growth and sporulation; 0, AIB factor ineffective (no growth).

Discussion

The acquisition of BR and IBR strains pointed to the possibility of reducing the sensitivity of *S. cinnamonensis* to these substances with the aim of positively influencing monensin A biosynthesis. Better yields of monensin A in submerged cultures with butyrate or isobutyrate were seen in 9% of BR and 3% of IBR strains. Nevertheless, the toxicity of both monensin A precursors was comparatively high and therefore their effect was not too significant. In addition, this insensitivity was found to be an unstable marker. A similar situation was also found with the propionate-resistant strains of *S. cinnamonensis* (Matějů *et al.*, 1988). As to the production of monensin A, more favourable results were obtained after feeding *S. cinnamonensis* with valine (Pospíšil *et al.*, 1989), a natural substrate and precursor of butyrate (Pospíšil *et al.*, 1983).

The biosynthesis with the highest proportion of the desired monensin A (90–95%) was found in regulatory mutants as a result of the overproduction of valine (Pospíšil *et al.*, 1984).

The most valuable result of this work was the finding that some IBR strains produce an unknown substance named the AIB factor. *S. cinnamonensis* genes encoding resistance to isobutyrate (or butyrate) and production of the AIB factor are probably not linked and accordingly the resistance gene(s) could be subject to instability independently of AIB factor production. It is known that genetic instability in streptomycetes is confined to localized areas of the genome (Cullum *et al.*, 1988).

Excretion of the AIB factor was originally observed in some IBR strains growing on agar medium with inhibitory concentrations of isobutyrate. Dwarf colonies which appeared very late on the agar medium containing 30 mM-isobutyrate did not exhibit production of the AIB factor. The possibility that these IBR strains not excreting the AIB factor suffer merely from a disturbance of membrane transport cannot be ruled out. Under non-restrictive culture conditions, i.e. without isobutyrate, the AIB factor was produced at a significant level and excreted into agar by wild-type *S. cinnamonensis* C-100-5. AIB factor production is probably independent of monensin biosynthesis because strain 10-IBR-9 excreting the AIB factor exhibited no monensin production.

The properties of the AIB factor are remarkable. *S. cinnamonensis* spores spread on agar medium containing toxic concentrations of salts of low molecular mass aliphatic monocarboxylic acids demonstrated no sign of growth. By adding the AIB factor, germination and growth started and the complete differentiation process accompanied by antibiotic production was realized. However, during submerged culture the AIB factor was not effective, and it exhibited an inhibitory action at higher concentrations.

It is plausible to suggest that the AIB factor is an important agent in the very early stages of germination on agar medium containing isobutyrate or its homologues. At some particular moment in the germination process it must be present either as the cell's own product or as a substance added from outside. Wild-type and IBR strains probably differ not only in the quantitative level of AIB factor production, but also in the timing of its biosynthesis. Differences in the activity of the AIB factor in surface and submerged cultures may be due to differences in metabolism and life-cycle under these conditions (Kalakoutskii & Agre, 1976).

The AIB factor seems to be a very effective signal molecule. Streptomycetes are well known to be producers of such substances, which are sometimes called autoregulators (Gräfe, 1989; Khokhlov, 1986; Sato *et al.*, 1989). The signal molecules described so far trigger normal

processes (formation of aerial mycelia, sporulation, antibiotic production) unlike the AIB factor which causes active protection against toxic, structurally similar, low molecular mass substances. It is not possible to exclude further pleiotropic functions of the AIB factor; for instance, stimulation of *S. cinnamonensis* sporulation was sometimes observed but was not reproducible. However, at present the suppression of the toxic effect of isobutyrate and homologous substances is the only unambiguous observed effect.

The AIB factor acts not only on *S. cinnamonensis*, but also on some other *Streptomyces* species. Production of the AIB factor of structurally related substances by other streptomycetes would be in accord with its being a signal molecule. For example, γ -butyrolactone-type autoregulators, such as A-factor and its derivatives, are widely distributed among the actinomycetes, and even γ -butyrolactone antibiotics are known (Gräfe, 1989).

The reason for the toxicity of acetate, propionate, butyrate, etc., has not been clarified so far. These carboxylic acids can enter cell metabolism after activation by coupling with CoA, catalysed by acyl-CoA synthetases or CoA-transferases (e.g. succinyl CoA, fatty acid CoA-transferase). This is probably the only common step in their metabolism. The AIB factor could indirectly influence this CoA activation, or it could participate at another level, e.g. it might affect membrane transport.

Attention is currently being paid to biochemical and morphological effects of butyrate on eukaryotic cells. Butyrate influences differentiation, synthesis of polypeptide hormones, glycoproteins or enzymes in several cell lines and induces hyperacetylation of histones (Kruh, 1982). In *Dictyostelium discoideum*, butyrate acts as an inducer of differentiation, while propionate and isobutyrate are less effective or have different effects on *D. discoideum* growth and development (Boto *et al.*, 1987). The present study indicates that in prokaryotic cells, represented in this case by streptomycetes, these substances can also, directly or indirectly, have a significant influence.

It will be important to determine the structure of the AIB factor, but at this stage of the investigation it was not possible to determine even the type of chemical structure of the AIB factor. The biological activity of the AIB factor might be exerted at very low concentrations and there is a danger of assigning the structure of an inactive substance to the AIB factor as happened in the case of the L-factor (Gräfe, 1989; Khokhlov, 1986).

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