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Short communication

A combined strategy to simultaneously improve the component of isovalerylspiramycin in a bitespiramycin (4″-*O*-acylspiramycins) complex and its biological titre

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ABSTRACT

Bitespiramycin is a group of 4″-*O*-acylated spiramycins with 4″-*O*-isovalerylspiramycins (ivSp) as the major components. It is a key problem how to selectively enhance the biosynthesis of ivSp components during fermentation process. The addition of isovalerate, a precursor supplier of 4″-side chain of ivSp, enhanced the ivSp content in the bitespiramycin complex, but reduced the biological titre owing to the inhibition of growth. At transition phase between growth stage and production stage, the addition with 2-oxoglutarate (2-OG) elevated the titre of bitespiramycin and took no effect on the composition. Combined feeding of isovalerate and 2-OG gave a marked increase in ivSp content accompanied a lift of biological titre. These results suggest that the combined use of the precursor provider and the regulator of carbon metabolism was a successful and cost-effective approach to achieve the balance of biosynthesis of bitespiramycin in terms of composition and titre.

1. Introduction

16-Membered macrolide antibiotic is a class of profound medicine for anti-infection [1]. Comparison of antimicrobial activities of the macrolide compounds with their derivatives acylated at mycarose position 4 indicates a clear structure–activity relationship: the more the number of carbon atom of the acyl group, the higher the biological activity [2–4]. Bitespiramycin (shengjimycin) is a group of 4″-*O*-acylated spiramycins with 4″-*O*-isovalerylspiramycins (ivSp) as the major components [5]. It was also proved that though bitespiramycin has similar antibacterial activity with spiramycin (Sp) *in vitro*, it has superior pharmacokinetic traits than spiramycin *in vivo* due to its high lipophilicity [6–8]. Phase III clinical research of bitespiramycin is undergoing.

Bitespiramycin was produced by genetically engineered *Streptomyces spiramyceticus* F21 transformed with 4″-*O*-acyltransferase gene from *S. mycarofaciens* 1748 [9]. There are more than 10 components in bitespiramycin owing to the relatively broad substrate spectrum of the 4″-*O*-acyltransferase (as shown in Fig. 1) [10]. So it is a key problem how to selectively enhance the biosynthesis of 4″-*O*-isovalerylspiramycins during the fermentation process of bitespiramycin.

It has been known that isovaleryl group is derived from leucine in microorganisms. And it is proved that the isovaleryl side chain of macrolide antibiotics, such as leucomycin, magnamycin as well as 3-*O*-acetyl-4″-*O*-isovaleryltylosin, is derived from leucine [11–13]. Though we also found that leucine feeding could improve the composition of bitespiramycin complex (data not shown), it is not feasible to use this approach in the commercial production because of the possible expensive cost. It usually was observed that the short-chain fatty acids derived from sugar metabolism or branched chain amino acids were secreted into the broth in the exponential phase and absorbed again by the microorganism to synthesize spiramycin at production phase [14]. So we tested the effect of exogenous isovalerate addition on the composition and titre of bitespiramycin. It was observed that the proportion of ivSp markedly increased, but the biological titre decreased.

In order to meliorate the decrease of titre at the condition of isovalerate feeding, 2-oxoglutarate (2-OG) was considered. 2-OG is derived from sugar metabolism, which involves glycolysis, tricarboxylic acid (TCA) cycle and anaplerotic pathway as well as amino acid transamination. It is known that 2-OG works as an important messenger molecule to coordinate carbon and nitrogen metabolisms in microorganisms [15,16]. In present study, we investigated the effects of isovalerate and 2-OG feeding alone or combined on the composition and titre of bitespiramycin and intended to explore an approach to enhance the ivSp content in the bitespiramycin complex based on that at least not to reduce the biological titre of bitespiramycin.

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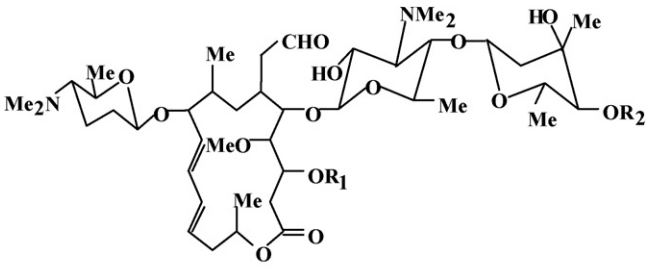

Components	R_1	R_2
Isovalerylspiramycins III	$COCH_2CH_3$	$COCH_2CH(CH_3)_2$
Isovalerylspiramycinsll	$COCH_3$	$COCH_2CH(CH_3)_2$
Isovalerylspiramycinsl	H	$COCH_2CH(CH_3)_2$
Isobutyrylspiramycin III	$COCH_2CH_3$	$COCH(CH_3)_2$
Isobutyrylspiramycinll	$COCH_3$	$COCH(CH_3)_2$
Butyrylspiramycin III	$COCH_2CH$	$COCH_2CH_2CH_3$
Butyrylspiramycinll	$COCH_3$	$COCH_2CH_2CH_3$
Propionylspiramycin III	$COCH_2CH_3$	$COCH_2CH_3$
Propionylspiramycinll	$COCH_3$	$COCH_2CH_3$
Acetylspiramycin III	$COCH_2CH_3$	$COCH_3$
Acetylspiramycin ll	$COCH_3$	$COCH_3$
Spiramycin III	$COCH_2CH_3$	H
Spiramycinll	$COCH_3$	H
Spiramycinl	H	H

Fig. 1. The structure of bitespiramycin.

2. Materials and methods

2.1. Microorganism and cultivation conditions

A genetically engineered strain WSJ-1 constructed by homologous recombination of a 4″-*O*-isovaleryltransferase gene from a carbomycin-producing strain *S. mycarofaciens* 1748 into the chromosome of *S. spiramyceticus* F21 [9], was used through this study.

The seed medium contained (g/l): glucose 1; corn starch 3; soybean meal 2; NaCl 0.4, $CaCO_3$ 0.5. *S. spiramyceticus* WSJ-1 spores were maintained in 20% Glycerol solution at −80 °C, and the concentration was more than 5×10^6 spore/ml. 1 ml spore solution was inoculated into a primary 250-ml shake flask containing 50 ml seed medium. After 48 h of incubation at 28 °C, 220 rpm, 10 ml primary culture was inoculated into a secondary 500-ml shake flask containing 100 ml seed medium. After 24 h of incubation at 28 °C and 220 rpm, 4 ml of secondary culture was inoculated into a fermentation flask.

The basal fermentation medium contained (g/l): corn starch 60; glucose 5; fish powder 20; yeast powder 5; ammonium nitrate 6; potassium phosphate 0.5; magnesium sulfate 1; sodium chloride 10; soybean oil 5. The culture was carried out with 500-ml flask containing 50 ml fermentation medium at 28 °C, 220 rpm for 4 days.

Isovaleric acid (5%) and 2-oxoglutaric acid (2.5%) concentrated solutions were prepared by neutralizing with 4N NaOH to pH 6.2–6.5 and autoclaved at 115 °C for 20 min. The addition of these organic acids to fermentation flasks was indicated in the text.

2.2. Analysis methods

2.2.1. Biomass concentration

Packed mass volume (PMV) was determined by centrifuging of 10 ml culture broth at 3000 rpm for 10 min.

2.2.2. Biological potency

This was done by the conventional disc diffusion method using *Bacillus pumilus* as a test strain and acetylspiramycin as standard.

2.2.3. Reduced sugar and total sugar in fermentation broth

The supernatant of fermentation broth after centrifugation was boiled with 6N HCl for 10 min and neutralized by equal volume of 6N NaOH. The above acid-hydrolysed solution was used for total sugar assay. The supernatant of fermentation broth after centrifugation was used for reduced sugar analysis. Sugar concentrations were determined by dinitrosalicylic acid (DNS) method [17].

2.2.4. Composition of bitespiramycin and short-chain organic acids in the broth

The high-performance liquid chromatography (HPLC) system (Agilent HP 1100) used here comprised of a multi-solvent delivery system, a G1328A injector and a G1314A variable wavelength detector (Agilent Tech, USA).

The components of bitespiramycin were detected by HPLC using a C_{18} column (TSKgel ODS-100S, 150 mm × 4.6 mm, 5 μm particle diameter) at 231 nm. The sample volume was 20 μl. Separation was achieved with a mixture of 1% NaH_2PO_4 and methanol (53:47) at a flow rate of 1 ml/min.

Short-chain fatty acids were quantified by HPLC using a C_8 column (AquaSep, 250 mm × 4.6 mm, 5 μm particle diameter) at 210 nm. The analysis was done with 10 mM phosphatic acid (adjusted to pH 2.2 with NaH_2PO_4) at a flow rate of 0.6 ml/min. The sample volume was 20 μl.

3. Results

3.1. Effect of isovalerate feeding on bitespiramycin composition and titre

In preliminary experiments, 0.5 g/l fed at 0 h, 6 h, 12 h, 18 h, 24 h, 30 h, 36 h and 0.1 g/l, 0.3 g/l, 0.5 g/l, 0.7 g/l, 0.9 g/l fed at 18 h, were tested, and 0.5 g/l isovalerate fed at 18 h at the middle of the exponential phase was verified to be the best option in the experimental conditions described in organism and cultivation conditions. As shown in Table 1, under this condition, total ivSp content increased by 15.5%, while total Sp decreased by 9%, which implied isovalerate could be absorbed by this microorganism and a marked shortage of isovaleryl group *in vivo*. The other acylated spiramycins also decreased obviously indicating a possible competition for the 4″ position of Sp between isovaleryl group and the acyl groups of other short-chain acids.

Fig. 2 illustrates the main parameters of the fermentation process with and without isovalerate feeding. The growth of cells became slower immediately after isovalerate addition at 18 h, and the biggest biomass was reduced by 20.2% showing a strong inhibitory effect. Correspondingly the total sugar consumption rate decreased and the pH level was always higher than the culture without isovalerate feeding. The biological titre of the culture with isovalerate supplementation was 86% of the control. Considering the decrement of biomass, it was implied that the isovalerate feeding did not decrease the specific synthesis ability of the cells. As the main 2-C precursor of bitespiramycin, the acetate level in the two cultures was similar to each other. But the level of propionate, a 3-C precursor, in culture with isovalerate feeding was markedly higher than the control. The concentration of succinate, another source of 3-C, exceeded that of the control after 30 h cultivation post-isovalerate addition.

3.2. Effect of 2-oxoglutarate feeding on bitespiramycin composition and titre

As depicted above, though isovalerate addition meliorated the composition of bitespiramycin complex to a large extent the biological titre decreased by 14% compared to the control, and sugar consumption rate seemed to be the most key parameter to

Table 1
Effect of 0.5 g/l isovalerate fed to culture at 18 h on the composition of bitespiramycin

Culture conditions	Component (%)		
	Total ivSp[a]	Total Sp[b]	Other acylated Sp[c]
Control	39.7 ± 0.7	24.8 ± 0.4	35.5 ± 0.9
Feeding isovalerate	55.2 ± 0.8	15.8 ± 0.2	29.0 ± 1.0

[a] Isovalerylspiramycins.
[b] Spiramycins.
[c] Other acylated spiramycin calculated by (100 − total ivSp − total Sp). Data are the mean ± S.E. (n = 3).