Selective Production of Capreomycins through Mutation and Medium Development

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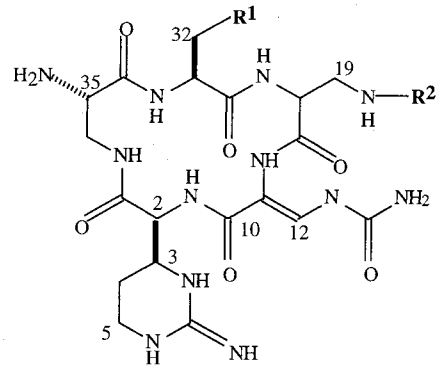
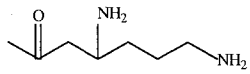
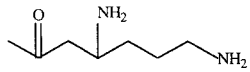
Streptomyces capreolus produces capreomycins IA, IB, IIA, and IIB[1]. These compounds are antitubercular antibiotics with activity against Gram-positive and Gram-negative bacteria[2]. Capreomycins of the I class have a β-lysine side chain while compounds of the II class lack this moiety (Fig. 1). The A capreomycins have a hydroxyl at R^1 from serine incorporation into the ring, whereas, the B capreomycins have a hydrogen at this position from alanine incorporation[1]. Through mutation and alteration of key medium components, selective production of each of the four components was achieved.

The strains isolated for selective capreomycin production are described in Table 1. These strains were fermented in defined media as follows: agar plugs of cultures were inoculated into 6 ml S-1 medium (1.5% dextrose, 0.5% Difco Bacto Tryptone, 0.25% Difco Yeast Extract, 0.3% $MgSO_4 \cdot 7H_2O$, 0.0001% $Fe_2(SO_4)_3$, and 0.0025% $CaCl_2$, pH adjusted to 7.0) in $1 \times 6''$ culture tubes with metal caps containing 2 glass beads (5 mm), using 6 mm diameter transfer pipets. All tube incubations were at 29°C, 225 rpm, at an approximately 4° angle. After 48 hours incubation, 0.4 ml of each culture was inoculated into 4 ml of Def-1 medium (6% dextrose, 0.3% L-asparagine, 0.075% $CaCl_2 \cdot 2H_2O$, 0.3% $MgSO_4 \cdot 7H_2O$, 0.0005% $Fe_2(SO_4)_3$, 0.04% KCl, and 0.02% Na_2HPO_4, pH adjusted to 7.0) in $1 \times 6''$ culture tubes with metal caps. Where indicated, L-serine or L-alanine was added to defined medium as a filter-sterilized, pH 7 stock solution. Tubes were incubated at 29°C, 225 rpm, at an approximately 4° angle for a total of 5 days.

Fig. 1. Structures of capreomycins IA, IB, IIA, and IIB.



Capreomycin	R^1	R^2
IA	OH	
IB	H	
IIA	OH	H
IIB	H	H

Mutants were selected by screening in a complex medium as described in the Experimental section. Using this method, *S. capreolus* CAP8-6 was selected for its ability to produce the II class of capreomycins preferentially. When this strain was fermented in a defined medium with or without supplemental L-alanine, capreomycin IIB was predominantly produced (Table 2). However, when L-serine was added to the fermentation, capreomycin IIA was the primary product.

S. capreolus CAP47-38 was chosen for its ability to produce capreomycin IA selectively. When this mutant was fermented in F10a production medium, it consistently produced a capreomycin IA to IB ratio of 28 to 1.

S. capreolus CAP7-75 was chosen for its ability to produce capreomycin IB preferentially (Table 3). When L-alanine was added to the defined medium, the IB to IA ratio was increased to 4 to 1. Adding L-serine to this medium shifted the ratio such that capreomycin IA was the predominant component.

We have demonstrated the capacity to produce

Table 1. Capreomycin production phenotype of mutant cultures.

Strain	Phenotype
S. capreolus CAP8-6	Produces capreomycin IIA and IIB
S. capreolus CAP47-38	Produces predominantly capreomycin IA
S. capreolus CAP7-75	Produces predominantly capreomycin IB

Table 2. Production profile of *S. capreolus* CAP8-6.

Medium	Capreomycin production ratio
Def-1	IIB to IIA ratio = 3 to 1
Def-1 + 0.8% L-alanine	IIB to IIA ratio = 5 to 1
Def-1 + 0.8% L-serine	IIA to IIB ratio = 3 to 1

Table 3. Production profile of *S. capreolus* CAP7-75.

Medium	Capreomycin production ratio
Def-1	IB to IA ratio = 2 to 1
Def-1 + 0.8% L-alanine	IB to IA ratio = 4 to 1
Def-1 + 0.8% L-serine	IA to IB ratio = 4 to 1

capreomycins IA, IB, IIA, or IIB selectively using mutation and the alteration of key medium components. Specifically, a mutant was selected that primarily produced the capreomycin II's. This organism could then be used to make predominantly IIA or IIB by adding serine or alanine, respectively. In addition, mutants were selected that primarily produced capreomycin IA or capreomycin IB.

Experimental

Ethyl Methanesulfonate (EMS) Mutagenesis

Strains to be mutagenized were stored in 20% glycerol at −20°C and revived on $^1/_2$YPD agar (0.5% Difco Yeast Extract, 0.5% Difco Bacto Peptone, 0.25% dextrose, 0.5% MOPS, and 1.7% Difco Bacto agar, pH adjusted to 7.0) at 28°C for 5 days. The cultures were then inoculated into 30 ml $^1/_2$YPD broth (0.5% Difco Yeast Extract, 0.5% Difco Bacto Peptone, 0.25% dextrose, and 0.5% MOPS, pH adjusted to 7.0) in a 300 ml Erlenmeyer flask and incubated at 29°C, 200 rpm, until reaching approximately $^1/_8$ to $^1/_4$ the turbidity of a fully grown culture. Two milliliters of the culture were aliquoted into each of 5 sterile culture tubes (17 × 100 mm). EMS (d = 1.17 g/ml) was added to the tubes (0, 10, 20, 30, or 40 μl), which were then incubated at 29°C, 220 rpm, for $4^1/_2$ hours. Following incubation, the cells were washed by adding 8 ml $^1/_2$YPD broth to each tube, centrifuging (5585 g) for 5 minutes, and carefully pipetting off the supernatant. The pellets were resuspended in 2 ml $^1/_2$YPD broth and plated for titer on $^1/_2$YPD agar to determine the degree of survival. Following titering, incubation was continued at 29°C, 220 rpm for 24 hours. Sterile 85% glycerol was added at 24 hours to a final concentration of 40%, and the mutagenized cultures were stored at −20°C.

Mutant Screening

EMS-mutagenized cells were plated for single colonies on $^1/_2$YPD agar and incubated at 28°C, 7 days. Agar plugs of colonies were then inoculated into 6 ml F10a medium (3% Nutrisoy flour (Archer Daniels Midland), 2% cerelose, 0.33% $MgSO_4 \cdot H_2O$, 0.1% $CaCO_3$, pH adjusted to 7.0) in 25 × 150 mm tubes with metal caps containing 2 glass beads (5 mm), using sterile 6 mm diameter transfer pipets. Cultures were incubated at 29°C, 225 rpm, at an approximately 4° angle. After 48 hours incubation, 0.4 ml of each culture was inoculated into 4 ml F10a medium in 1 × 6″ tubes with metal caps, then incubated at 29°C, 225 rpm, at an approximately 4° angle for 5 days.

Production stage cultures were harvested by adding 0.5 ml whole broth to 9.5 ml CAP mobile phase (0.54 M NaCl in 0.05 M acetic acid-acetonitrile, 80% : 20%). Samples were mixed and centrifuged at 1273 g for 20 minutes for HPLC analysis.

(Note: All media were prepared in distilled water.)

HPLC Analysis

Chromatography was carried out using a Thermo Separation Products pump (cM4000) and detector (sM3100). 20 μl samples were injected onto a Mono S HR 5/5 column at 30°C, eluted at a flow rate of 1.2 ml/minute, and monitored at 270 nm.

Acknowledgments

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