RIBOSTAMYCIN PRODUCTION BY A MUTANT OF BUTIROSIN PRODUCING BACTERIA

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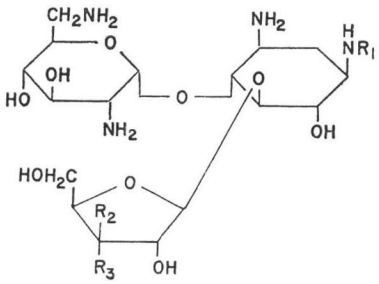
By the use of our improved colony selection technique, xylostasin and ribostamycin producing mutants were isolated from nitrosoguanidine treated *Bacillus circulans* B15M, a producer of butirosins A and B. Among these structurally related aminoglycosides, ribostamycin is the well-known product of a *Streptomyces* and has not been isolated as a bacterial metabolite.

A selected mutant of strain 306, which produces xylostasin and ribostamycin, was further mutagenized in expectation of getting an improved strain having the ability to accumulate a large amount of ribostamycin in the culture broth. One mutant, strain 451, derived from strain 306, produced ribostamycin free of xylostasin.

Butirosins A and B, xylostasin and ribostamycin are structurally related aminoglycoside antibiotics (Fig. 1). Among these, ribostamycin (deacylated butirosin B) is solely the product of a *Streptomyces*[1] and has been in clinical use because of its relatively low toxicity and wide range of activity against Gram-positive and Gram-negative bacteria.

Xylostasin (deacylated butirosin A), on the other hand, is the product of a *Bacillus* sp.[2] Since butirosins A and B are also products of *Bacillus circulans*,[3] we tried to get a ribostamycin-producing mutant from a butirosins producing *B. circulans*, using the "cylinder culture method" and the "double indicator method." We report here on a simple screening system for mutant isolation and the ribostamycin production by the isolated mutant.

Fig. 1. Structure of butirosins A and B, xylostasin and ribostamycin.



	R_1	R_2	R_3
Butirosin A	$COCHCH_2CH_2NH_2$ (OH)	OH	H
Butirosin B	$COCHCH_2CH_2NH_2$ (OH)	H	OH
Xylostasin	H	OH	H
Ribostamycin	H	H	OH

Materials and Methods

Strains

Bacillus circulans B15M: Butirosins producing strain, which was isolated from soil and identified by us.

Escherichia coli NIHJ JC2: Sensitive to butirosins, xylostasin and ribostamycin.

Escherichia coli 80750: Sensitive to butirosins but resistant to xylostasin and ribostamycin (MICs 100 mcg/ml).

Proteus vulgaris CN 329: Sensitive to xylostasin and ribostamycin (MICs 3.13 mcg/ml).

Serratia marcescens ATCC 13880: Sensitive to xylostasin (MIC 3.13 mcg/ml) but somewhat resistant to ribostamycin (MIC 25 mcg/ml).

Bacillus subtilis PCI 219: Sensitive to all antibiotics described above and used for bioautography.

Mutagenesis

To a log phase culture in TY medium (tryptone 1%, yeast extract 0.2%, NaCl 0.5%) at 37°C was added 200 mcg/ml of N-methyl-N′-nitro-N-nitrosoguanidine (NTG) and the mixture incubated for 30 minutes, at 37°C. Cells were washed by centrifugation and resuspended in TY medium. The overnight culture was diluted and plated onto a TY agar plate.

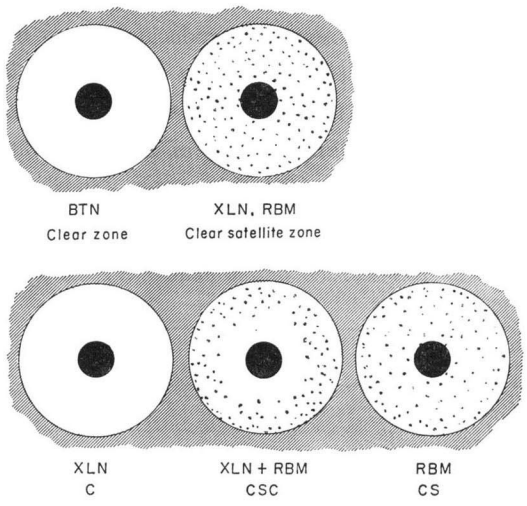
Cylinder culture method

Eighty ml of production agar medium containing 1% glycerol, 2% Soytone, 0.3% $MgSO_4 \cdot 7H_2O$, 2.5% agar was autoclaved and poured into a rectangular plastic dish (8×23 cm), and then 120 glass cylinders (10 mm in inside diameter, 10 mm in height) were placed in a row into the molten medium. Colonies grown on TY agar plates were inoculated in cylinders by picks. Cylinder cultures were incubated at 28°C for 5 days.

Double indicator assay method (Fig. 2)

Nutrient agar medium was inoculated with 10^6 cells/ml *E. coli* JC2 and 10^3 cells/ml *E. coli* 80750. On this assay plate, butirosin impregnated paper discs or butirosin producing cylinder cultures form clear inhibition zones (C zones), and xylostasin or ribostamycin form clear satellite zones (CS zones). This is because a small number of *E. coli* 80750 cells grow in an inhibition zone of *E. coli* JC2 as satellite colonies. By this method, butirosin producing strains, xylostasin and/or ribostamycin producing strains and antibiotic non-producing strains can be distinguished from each other on one assay plate. To distinguish ribostamycin from xylostasin, a nutrient agar medium containing 10^6 cells/ml *Proteus vulgaris* CN329 and 10^3 cells/ml *Serratia marcescens* ATCC 13880 was used. Xylostasin forms a C zone, while ribostamycin forms a CS zone. The mixture of xylostasin and ribostamycin forms a clear satellite-clear zone (CSC zone), and as the xylostasin proportion in the mixture increases, the diameter of inside clear zone proportionately increases.

Fig. 2. Double indicator method.
Upper: 10^6 cells/ml of *E. coli* JC2 and 10^3 cells/ml of *E. coli* 80750 were inoculated in nutrient agar.
Lower: 10^6 cells/ml of *Pr. vulgaris* CN 329 and 10^3 cells/ml of *Ser. marcescens* ATCC 13880 were inoculated in nutrient agar.
BTN: butirosin, XLN: xylostasin, RBM: ribostamycin.



Fermentation

Seed medium containing 2% soluble starch, 1% tryptone (Difco), 1% yeast extract (Difco), 0.3% $MgSO_4 \cdot 7H_2O$, pH 7.0 was inoculated with a loopful of cells grown on a soybean agar slant, and incubated at 28°C for 24 hours on a reciprocal shaker. Four ml of the seed culture was transferred into 100 ml of fermentation medium containing 2% corn starch, 3% soybean meal, 0.3% $MgSO_4 \cdot 7H_2O$, and 1% $CaCO_3$. After 5 days incubation at 28°C on a reciprocal shaker, antibiotic production reached a maximum. A 30-liter jar fermentation was carried out with the same seed medium and a fermentation medium containing 3% corn starch, 2% soybean meal, 1% fish meal, 0.3% $MgSO_4 \cdot 7H_2O$, and 1% $CaCO_3$. The fermentation was aerated at 1.0 volume of air per volume of medium per minute and agitated at 350 rev/min for 5 days at 28°C.

TLC

Chromatographic comparison of xylostasin, ribostamycin and the butirosins was performed by silica gel thin-layer chromatography (Tokyo Kasei); solvent A, chloroform – methanol – 28% ammonia (1: 3: 2); solvent B, chloroform – methanol – 28% ammonia – water (1: 4: 2: 1); solvent C, chloroform – methanol – 17% ammonia (2: 1: 1, upper phase). Xylostasin and ribostamycin were separated by TLC with an alumina plate developed by solvent C, or by silica gel TLC, dipped in 0.2% boric acid and reactivated before use, developed by solvent C.

Results and Discussion

Three mutant strains, 305, 306 and 308, were isolated from about 2,600 NTG treated colonies of *B. circulans* B15M. TLC bioautograms indicated that strains 305 and 308 produced xylostasin exclusively and strain 306 produced ribostamycin and xylostasin in a ratio of 2: 8 in a flask experiment. A jar fermentation with strain 306 decreased the ratio to 1: 85. We therefore sought a further mutant of strain 306 which would produce larger quantities of ribostamycin. Unfortunately, an additional mutation occurred in strain 306 which resulted in the formation of spreading colonies. Since this was disadvantageous for later mutation experiments, strain 306 was treated with NTG to yield a new mutant, Mot 3, which formed more desirable round colonies.

Shikimic acid requiring mutants and galactose non-utilizing mutants were isolated from the strain Mot 3 to increase ribostamycin production, the former to accumulate ribose[4], and the latter to disturb the sugar metabolic pathway. However none of these mutants showed increased ribostamycin production. In the course of this screening, mutant strain G20 was isolated from strain Mot 3. This mutant produced xylostasin at high titre but no ribostamycin. We used strain G20 as a parent for the screening of a ribostamycin-producing mutant by the cylinder culture and double indicator method which distinguishes ribostamycin from xylostasin. Two colonies were isolated from about 1,600 tested. Strain 251 was isolated as a CSC zone producer, while strain 451 was isolated as a CS zone producer. TLC-bioautography of the culture filtrates showed that xylostasin and ribostamycin were produced in a ratio of 4: 5 by strain 251 whereas strain 451 produced ribostamycin exclusively as expected from the CS zone formed on the double indicator assay plate.

Table 1. Identification of the antibiotic produced by strain 451.

	Ribostamycin standard	Antibiotic from strain 451
$[\alpha]_D^{22}$ (c 1, H_2O)	+42.2°	+42.4°
Elemental analysis for $C_{17}H_{34}N_4O_{10}$		
Calcd. C 44.92	Found: 44.48	Found: 44.45
H 7.54	7.50	7.69
N 12.33	12.05	11.98
NMR (in D_2O)	In agreement	
TLC by silica gel Solvent* A	Rf 0.17	0.19
B	0.33	0.34
C	0.59	0.60
Acid hydrolysis (1.5 N HCl, 100°C 2 hours)	Ribose	Ribose

* See Methods.

Table 2. Antibacterial spectrum of antibiotic from strain 451.

	MIC (mcg/ml)	
	Ribostamycin standard	Ribostamycin from 451
Escherichia coli ATCC 25922	3.13	3.13
Escherichia coli NIHJ JC-2	3.13	3.13
Proteus vulgaris ATCC 6380	0.78	0.78
Staphylococcus aureus 209P	0.78	0.78
Staphylococcus aureus ATCC 25923	1.56	1.56
Bacillus subtilis PCI 219	0.78	0.78
Sarcina lutea PCI 1001	50	50

MIC: Minimal inhibitory concentration in MUELLER-HINTON agar dilution method.

A jar fermentation of strain 451 produced ribostamycin at levels of 825 mcg/ml (sulfate salt).

The antibiotic was isolated by usual techniques from a 30-liter jar fermented broth of strain 451. The isolate was identical to an authentic sample of ribostamycin with respect to optical rotation, elemental analysis, IR, NMR, GC, MS, HPLC and antibacterial spectrum. Mild acid hydrolysis liberated ribose but no xylose. The data is summarized in Tables 1 and 2. Fermentation of strain 306 yielded two antibiotics which were isolated and identified as ribostamycin and xylostasin.

Physical, chemical and biological identification indicates that strain 451 is a ribostamycin-producing mutant of *B. circulans*. This is the first example of an organism, obtained by mutation and belonging to the family *Bacillaceae*, that produces an aminoglycoside antibiotic of *Streptomyces* origin, and implies that other types of antibiotics from *Streptomyces* could also be produced by other bacteria.

We suggest that xylostasin and ribostamycin are the intermediates in the biosyntheses of butirosins A and B. Recently TAKEDA *et al.*[5] proposed that ribostamycin is an intermediate in the butirosin biosynthetic pathway. Ribostamycin is also proposed as an intermediate in the neomycin biosynthesis in *Streptomyces fradiae*.[6] There may be a common origin of genes that are responsible for the production of 4,5-substituted 2-deoxystreptamines.

The "cylinder culture method" is similar to the agar piece method used by ICHIKAWA *et al.*,[7] but our method is freer of contamination and does not require a humid chamber. The "double indicator method" has the advantage of picking up exactly the desired mutants from many low- and non-producing mutants. These methods offer a simple and effective procedure for the improvement of antibiotic producing strains.

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