ISOLATION OF A *STREPTOMYCES ROCHEI* IDIOTROPH REQUIRING β-LYSINE FOR PRODUCTION OF STREPTOTHRICIN

KARTAR SINGH, S. N. SEHGAL, S. RAKHIT and CLAUDE VÉZINA*

Department of Microbiology, Ayerst Research Laboratories, Montreal, Quebec, Canada

(Received for publication April 30, 1983)

A streptomycete (B-1115), isolated from an Easter Island soil sample and identified as *Streptomyces rochei*, produced a streptothricin complex[1]. The main component (AY-24,546) of the complex was isolated and characterized as racemomycin A[2] (streptothricin F; the simplest member of the group of usual streptothricins) by comparison with an authentic sample. KHOKHLOV has written a comprehensive review on streptothricins[3].

Attempts were made to obtain mutants that would synthesize AY-24,546 exclusively or predominantly or produce new antibiotics when fed a particular moiety of the antibiotic. Mutational biosynthesis (mutasynthesis) first described by SHIER *et al.*[4] was selected as a method which could give the required compounds.

Fifteen mutants were isolated and characterized as idiotrophic for the constituents of streptothricin. β-Lysine idiotroph B-1115/40 failed to produce streptothricin unless it was fed β-lysine. β-Lysine was specific for restoring antibiotic production and could not be replaced by any of the α or β amino acids or the α,β-diamino acids tested. Isolation of this idiotroph (B-1115/40) is reported in this communication. Direct incorporation of β-lysine into streptothricin by this idiotroph was also demonstrated, confirming the earlier observation of VORONINA *et al.*[5]

The morphological, cultural and physiological characteristics of producing organism B-1115 were studied according to ISP methods[6]. By comparison to species described in BERGEY's Manual of Determinative Bacteriology[7], organism B-1115 was identified as *Streptomyces rochei*.

S. rochei B-1115 was grown on tomato paste-oatmeal agar slants at 25°C for seven days. Spores were suspended in distilled water; the suspension was diluted to contain 5×10^7 spores per ml, then UV irradiated to ≥99% kill. Colonies of survivors were uniformly streaked on 100-mm Petri plates containing 20 ml of medium A (Bacto peptone 0.5%, dextrose 2%, NaCl 0.5%, Bacto agar 2%, pH 7.2). After six days of incubation at 25°C, agar plugs (7-mm diameter) were transferred onto Bacto-Antibiotic Medium 5 plates seeded with 1×10^8 spores of *Bacillus subtilis*. After incubation for 18 hours at 37°C, streptomycete colonies that were not surrounded by a inhibition zone were picked, purified twice on medium A and retested against *B. subtilis*. Sixteen streptomycete mutants which exhibited no antibiotic activity were selected.

The sixteen presumptive mutants as well as the parent strain B-1115 were streaked uniformly on 100-mm Petri plates containing 20 ml of medium A supplemented with 200 or 400 μg/ml of streptolidine, D-glucosamine (D-gulosamine was not available; we therefore used D-glucosamine which has been indicated to be a direct precursor for D-gulosamine moiety in racemomycin A[8]) or β-lysine, or combination thereof. After four days of incubation at 25°C, agar plugs were transferred on to Bacto-Antibiotic Medium 5 plates seeded with *B. subtilis*. Inhibition zones were measured after 18 hours of incubation at 37°C.

Only four mutants responded. The results are (Table 1): B-1115/31 was idiotrophic for all three moieties, B-1115/4 and B-1115/38 were idiotrophic for β-lysine and streptolidine; only B-1115/40 was idiotrophic for only one moiety, β-lysine. None of the 12 other blocked mutants responded to any of the three moieties, or combination thereof (not shown in Table 1). The addition of streptothricin moieties produced no observable effect on growth of either the parent strain or the mutants. None of the streptothricin moieties inhibited *B. subtilis* or affected the formation of streptothricin by parent B-1115.

S. rochei (B-1115) was cultivated in 500-ml Erlenmeyer flasks each containing 100 ml of medium B that contained (g/liter): Bacto peptone 10, dextrose 10, Hunt's tomato paste 2, NaCl 2.5, "blackstrap molasses" 4, pH 7.5. The flasks were inoculated with a spore suspension and incubated on a gyrotary shaker (240 rev/

* Present address: Institut Armand-Frappier, 531, boulevard des Prairies, Ville de Laval, Que., Canada H7V 1B7

Table 1. Influence of D-glucosamine, β-lysine and streptolidine on streptothricin synthesis by idiotrophs of *S. rochei* B-1115[a].

Idiotroph No.	No. addition	D-Glucosamine		β-Lysine		Streptolidine	
		200 μg/ml	400 μg/ml	200 μg/ml	400 μg/ml	200 μg/ml	400 μg/ml
Uninoculated	—	—	—	—	—	—	—
B-1115 (Parent)	21	21	21	21	21	21	21
B-1115/4	—	—	—	11	—	8	12
B-1115/31	—	—	12	14	18	—	16
B-1115/38	—	—	—	8	11	—	9
B-1115/40	—	—	—	18	20	—	—
Other 12 mutants	—	—	—	—	—	—	—

[a] Inhibition zones are in mm (7-mm agar plugs); test organism: *B. subtilis*.
—: no inhibition zone around the agar plug.

minute) at 25°C. The production of antibiotic started at 24 hours and rose to the maximum value of 1,000 μg/ml by 72 to 96 hours. Antibiotic activity was determined by a cylinder-plate assay using *B. subtilis* as a test organism.

For isolation of the antibiotic the broth filtrate was applied to an Amberlite IRC-50 (H^+) column. The column was washed with water and 0.1 N acetic acid and the antibiotic complex eluted with 0.3 N HCl, was neutralized and concentrated to a small volume. Excess of salt was removed by precipitation with methanol and the filtrate was chromatographed on Sephadex G-10. Fractions containing the antibiotic were pooled and applied to a column of CM-Sepharose C1-6B (NH_4^+). The column was eluted with NH_4HCO_3 using a concentration and pH gradient. AY-24,546 was eluted with 0.1 M NH_4HCO_3, pH 8.5.

The antibiotic was further purified by chromatography on carboxymethyl cellulose[9] (Whatman CM-52), using pyridine - acetic acid, pH 5.0 for development[2] or by preparative paper chromatography using 1-propanol - pyridine - acetic acid - water (15: 10: 3: 12). Antibiotic-containing fractions were pooled, converted into the hydrochloride by treatment with HCl (in methanol) and chromatographed on Sephadex LH-20[10] (methanol - water, 1: 9). The purified antibiotic (AY-24,546) obtained was detected as a single spot by TLC on silica gel and cellulose and paper chromatograms[11]. The Rf values of AY-24,546 were comparable to those of racemomycin A[2]; $[\alpha]_D^{25}$ −45.7° (*c* 1.0, H_2O); *Anal* calcd. for $C_{19}H_{34}N_8O_5 \cdot 3HCl \cdot H_2O$: C 36.22, H 6.20, N 17.79, Cl 16.92; found: C 36.29, H 6.10, N 17.82, Cl 15.72. Analyses of the acid hydrolysates of AY-24,546 and racemomycin A gave the same ratio of β-lysine to streptolidine[12] (approximately 1: 1). D-Gulosamine was identified as the amino sugar moiety of AY-24,546[13].

[*β-Lysine*-^{14}C]AY-24,546 was obtained by cultivating *S. rochei* B-1115 in shaken cultures to which L-[*U*-^{14}C]lysine was added at 30 hours after inoculation. After further incubation for 48 hours the fermentation was terminated and the antibiotic isolated and purified. The antibiotic was hydrolyzed with 6 N HCl at 115°C. TLC and radioautography of the hydrolysate showed that radioactivity was exclusively in the β-lysine moiety. β-[^{14}C]Lysine was isolated in almost quantitative yield by chromatography on CM-Sephadex C-25 (Fig. 1) using 0.1 M ammonium acetate for elution; 6% of the added L-lysine radioactivity was incorporated into β-lysine. This indicated that L-lysine was transformed into β-lysine during streptothricin biosynthesis by *S. rochei* as reported previously by VORONINA *et*

Fig. 1. Isolation of β-lysine and streptolidine by fractionation of AY-24,546 hydrolysate on CM-Sephadex C-25 column.
Elution: 0.1 M ammonium acetate, pH 7.0.

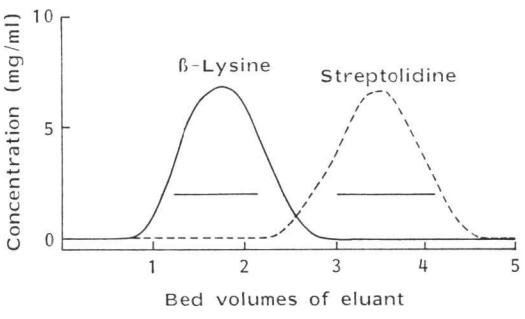

Table 2. Effect of time of β-lysine addition on streptothricin production in idiotroph B-1115/40.

β-Lysine added at (hours)	Streptothricin (μg/ml) produced at (hours)						
	0	18[a]	42	66	90	114	138[b]
0	0	0	0	0	0	0	11.5
18	0	0	0	35	390	850	>1,000
42	0	0	0	81	630	650	>1,000
66	0	0	0	0	150	300	>1,000
90	0	0	0	0	0	50	210
42 (L-Lysine)	0	0	0	0	0	0	0

[a] pH dropped to 5.0~6.5 and remained constant until the end of fermentation.

[b] Mycelial dry weight was 2.5~3.0 mg/ml at 138 hours of incubation.

Table 3. Effect of β-lysine concentration on streptothricin production by idiotroph B-1115/40.

β-Lysine added to culture medium at 48 hours (μg/ml)	Antibiotic produced (μg/ml)		
	24 hours[a]	48 hours	72 hours
0	0	0	0
100	320	925	188
200	450	1,000	380
400	655	1,000	—
600	445	760	335
800	280	735	235
1,000	445	735	260

[a] Incubation period after addition of β-lysine.

al.[14,15] and SAWADA *et al.*[16,17] for other streptothricin-producing organisms.

Idiotroph B-1115/40 did not produce antibiotic unless β-lysine was added to the growth medium. Idiotroph B-1115/40 grown in shake flasks containing liquid medium A (no agar) was fed β-lysine at 400 μg/ml final concentration at the times listed in Table 2. The results presented in Table 2 show that addition of β-lysine between 18 and 66 hours of incubation produced maximal streptothricin activity. Addition at time 0 led to very low yields; when added before the condensing enzymes were present, β-lysine was probably degraded instead of being incorporated into the antibiotic.

The experiment was repeated and the time of β-lysine addition was set at 48 hours after inoculation. β-Lysine was added at the final concentrations listed in Table 3. Samples were taken 24, 48 and 72 hours after the addition of β-lysine, and the concentration of streptothricin produced was measured. The results shown in Table 3 indicate that the best range of β-lysine concentration was 200~400 μg/ml for a 48-hour incubation. The maximal yield was 1,000 μg streptothricin equivalent per ml. The antibiotic isolated and purified was identified as AY-24,546 (streptothricin F). As shown in Table 2, β-lysine cannot be spared by L-lysine. Therefore, the genetic lesion in idiotroph B-1115/40 probably concerns its incapacity to transform L-lysine into β-lysine. Many attempts were made (not shown in Table 2) to replace β-lysine: glycine, formiminoglycine and several β-amino and α,β-diamino acids were found incapable of restoring antibiotic activity in the idiotroph.

The high specificity of the idiotroph for β-lysine is unusual, since it has been reported that enzymes involved in secondary metabolism are not very specific for their substrates[18]. Since only unlabeled compounds were studied, it remains possible that some were incorporated, but only inactive substituted products were synthesized.

The enzyme lysine 2,3-aminomutase, which catalyses the conversion of L-lysine to β-lysine, has been reported in *Clostridium* sp. SB4[19,20]. This pathway may be present in the parent strain of *S. rochei*. The idiotroph apparently lacks the enzyme lysine 2,3-aminomutase. However, we could not detect this enzyme in washed cells, protoplasts or cell-free extracts of parent strain B-1115. SAWADA *et al.*[16] also were unable to detect this enzyme in any of the streptothricin-producing strains of streptomycetes. In these organisms, the enzyme may be unstable.

Since an idiotroph that required β-lysine to produce streptothricin was available it offered an opportunity to assess whether β-lysine was directly incorporated into the antibiotic by this organism. β-[^{14}C]Lysine (20 mg, 1.7×10^{6} cpm/flask) was added to five Erlenmeyer flasks (500-ml flasks, each containing 100 ml of liquid medium A), and fermentation carried out as described previously. The antibiotic produced was isolated and purified. The labeled antibiotic showed an incorporation of 7% (total of 6×10^{5} cpm) of the β-lysine radioactivity added. TLC and radioautography of the acid hydrolysate showed the radioactivity to be exclusively in the β-lysine moiety. These results indicate that β-lysine is directly incorporated into the antibiotic by this organism. Direct incorporation of β-

lysine into polymycin (a streptothricin) has been reported by VORONINA *et al.*[5]

Acknowledgments

We are grateful to Dr. Y. SAWADA for providing us with a sample of racemomycin A. Technical assistance of Ms. G. MASSÉ, Ms. A. RICHARD and Mr. S. NGO is gratefully acknowledged.

References

1) WAKSMAN, S. A. & H. B. WOODRUFF: Streptothricin, a new selective bacteriostatic and bactericidal agent, particularly active against Gram-negative bacteria. Proc. Soc. Exp. Biol. Med. 49: 207~210, 1942
2) TANIYAMA, H.; Y. SAWADA & T. KITAGAWA: Characterization of racemomycins. Chem. Pharm. Bull. 19: 1627~1634, 1971
3) KHOKHLOV, A. S.: Streptothricins and related antibiotics. *In* Antibiotics: Isolation. Separation and Purification. *Eds.*, J. WEINSTEIN & G. H. WAGMAN, pp. 617~713, Elsevier Sci. Pub. Com., Amsterdam, 1978
4) SHIER, W. T.; K. L. RINEHART, Jr. & D. GOTTLIEB: Preparation of four new antibiotics from a mutant of *Streptomyces fradiae*. Proc. Natl. Acad. Sci. U.S. 63: 198~204, 1969
5) VORONINA, O. I.; I. I. TOVAROVA & A. S. KHOKHLOV: Incorporation of β-lysine into the polymycin molecule. Izv. Akad. Nauk. SSSR, Ser. Biol. 1969: 152~154, 1969
6) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
7) PRIDHAM, T. G. & H. D. TRESNER: *Streptomyces* Waksman and Henrici. *In* BERGEY's Manual of Determinative Bacteriology. *Eds.*, R. E. BUCHANAN & N. E. GIBBONS. pp. 748~829, The Williams and Wilkins Co., Baltimore, 1974
8) SAWADA, Y.; S. NAKASHIMA & H. TANIYAMA: Biosynthesis of streptothricin antibiotics. III. Incorporation of D-glucosamine into D-gulosamine moiety of racemomycin-A. Chem. Pharm. Bull. 25: 1478~1481, 1977
9) KHOKHLOV, A. S. & P. D. RESHETOV: Chromatography of streptothricins on carboxymethylcellulose. J. Chromatogr. 14: 495~496, 1964
10) TANIYAMA, H.; Y. SAWADA & T. KITAGAWA: Chromatography of racemomycins on dextran gel. J. Chromatogr. 56: 360~362, 1971
11) HOROWITZ, M. I. & C. P. SCHAFFNER: Paper chromatography of streptothricin antibiotics. Differentiation and fractionation studies. Anal. Chem. 30: 1616~1620, 1958
12) EGROV, C. A.; P. D. RESHETOV & A. S. KHOKHLOV: A method for quantitative analysis of streptothricin hydrolysates. J. Chromatog. 19: 214~215, 1965
13) STOFFYN, P. J. & R. W. JEANLOZ: Hyaluronic acid and related substances. XII. Identification of amino sugars by paper chromatography. Arch. Biochem. Biophys. 52: 373~379, 1954
14) VORONINA, O. I.; I. I. TOVAROVA & A. S. KHOKHLOV: Transformation of L-lysine into β-lysine during polymycin biosynthesis. Izv. Akad. Nauk. SSSR, Ser. Biol. 1973: 228~232, 1973
15) VORONINA, O. I. & A. S. KHOKHLOV: Pathway of biosynthesis of peptide moieties of streptothricin antibiotics. Postepy Hig. Med. Dosw. 26: 541~548, 1972
16) SAWADA, Y.; S. NAKASHIMA & H. TANIYAMA: Biosynthesis of streptothricin antibiotics. VI. Mechanisms of β-lysine and its peptide formation. Chem. Pharm. Bull. 25: 3210~3217, 1977
17) SAWADA, Y.; T. KUBO & H. TANIYAMA: Biosynthesis of streptothricin antibiotics. I. Incorporation of ^{14}C-labeled compound into racemomycin A and distribution of radioactivity. Chem. Pharm. Bull. 24: 2163~2167, 1976
18) QUEENER, S. W.; O. K. SEBEK & C. VEZINA: Mutants blocked in antibiotic synthesis. Ann. Rev. Microbiol. 32: 593~636, 1978
19) COSTILOW, R. N.; O. M. ROCHOVANSKY & H. A. BARKER: Isolation and identification of β-lysine as an intermediate in lysine fermentation. J. Biol. Chem. 241: 1573~1580, 1966
20) CHIRPICH, T. P.; V. ZAPPIA, R. N. COSTILOW & H. A. BARKER: Lysine 2,3-aminomutase. Purification and properties of a pyridoxal phosphate and *S*-adenosylmethionine-activated enzyme. J. Biol. Chem. 245: 1778~1789, 1970