DISCOVERY, PRODUCTION, AND BIOLOGICAL ASSAY OF AN UNUSUAL FLAVENOID COFACTOR INVOLVED IN LINCOMYCIN BIOSYNTHESIS

JOHN H. COATS, GRACE P. LI, MING-S. T. KUO and DAVID A. YUREK

Chemical and Biological Screening, The Upjohn Company, Kalamazoo, Michigan 49001, U.S.A.

(Received for publication September 21, 1988)

In a genetic study of *Streptomyces lincolnensis*, a riboflavin auxotroph, NTG-3, was obtained which was also a lincomycin non-producer under standard antibiotic production conditions. The addition of small amounts of sterile-filtered fermentation broth from a second non-producing *S. lincolnensis* mutant, NTG-5, fully restores the ability of NTG-3 to produce lincomycin. This response of the mutant NTG-3 was exploited in a biological assay to facilitate the isolation of "lincomycin cosynthetic factor" (LCF) found in the mutant NTG-5 and to determine in which part of the lincomycin biosynthesis pathway the factor functions. The isolation and characterization of the LCF from *S. lincolnensis* is the subject of the following note.

The parent lincomycin producing strain used in this study was *S. lincolnensis* (UC 5124), a derivative of the original lincomycin producing strain (UC 2376) isolated at The Upjohn Company.[1] NTG-3 (UC 8292) is a tightly blocked auxotroph derived from UC 5124. It grows well in riboflavin containing media, but does not produce significant amounts of lincomycin in the standard fermentation medium used in this study. NTG-5 (UC 8293), also derived from UC 5124, is a lincomycin non-producer but is not an auxotroph. NTG-5 has not been shown to make lincomycin under any fermentation condition. The *S. lincolnensis* mutants NTG-3 and NTG-5 were obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) treatment and characterized following the procedures employed by COATS and ROESER.[2]

For the fermentation of NTG-3 and NTG-5 in liquid culture, a seed medium was employed consisting of Yeastolac (Vico Products, Decatur, IL; 10 g/liter), NZ-Amine B (Sheffield Chemical, Norwich, N.Y.; 5 g/liter) and glucose monohydrate (Cerelose; 20 g/liter). Presterilization pH of the medium was adjusted to 7.2. A lincomycin production medium, F-13, was employed consisting of glucose monohydrate (15 g/liter), black-strap molasses (Pacific Molasses Co., San Francisco, CA; 20 g/liter), Hi-starch (Lauhoff Grain Co., Danville IL; 40 g/liter), cotton-seed hydrolysate (Pharmamedia, Traders Oil Mill Co., Fort Worth, Texas; 25 g/liter), $CaCO_3$ (8 g/liter), and K_2SO_4 (2 g/liter). Antifoam (Ucon LB625, Union Carbide Company) was added at the rate of 3 drops per 100 ml medium. Presterilization pH of the fermentation medium was also adjusted to 7.2. Seed cultures and fermentations were incubated at 28°C on a rotary shaker for 72 and 96 hours, respectively. For the assay of the LCF, 96-hour fermentation beers of NTG-3 were pooled and dispensed aseptically into sterile 125-ml Erlenmeyer flasks, 20 ml/flask. The flasks were frozen and maintained above liquid nitrogen until use. To assay fermentation broths or extracts for cosynthetic factor activity, 0.2 ml or less sterile filtered broth or extract was added directly to frozen assay flasks. The flasks were incubated for 18 hours on a rotary shaker at 28°C and assayed for lincomycin using a *Micrococcus luteus* disc-plate agar assay.[3]

During the course of LCF isolation/characterization studies, it became apparent that LCF was related structurally to riboflavin. The factor was subsequently determined to be 7,8-didemethyl-8-hydroxy-5-deazariboflavin. This compound has previously been identified as the chromophoric coenzyme F_{420} fragment (FO) in methanogenic bacteria which participates in NADPH mediated reductions[4] and as cosynthetic factor 1 in *Streptomyces aureofaciens* where it is involved in the reduction of 5a,11a-dehydrochlortetracycline to chlortetracycline.[5] Coenzyme F_{420} has also been found in fermentations of *Streptomyces griseus* presumably functioning in a photoreactivation system.[6]

Since the LCF was determined to be a riboflavin analog, it was tested for its ability to replace riboflavin for growth of the NTG-3 mutant on a minimal medium. No growth occurred when the factor was substituted for riboflavin. Thus, since riboflavin and the

Table 1. Effect of PPL and MTL additions to NTG-3 fermentations.

Addition to NTG-3 assay fermentation	Zone vs. *Micrococcus luteus* (12.5 mm disc; 80 μl)
PPL (200 μg/ml)	39 mm
MTL (200 μg/ml)	Trace
No addition	Trace

PPL and MTL were added to NTG-3 assay flasks and lincomycin production measured after 18 hours incubation by the *M. luteus* agar disc-plate assay.

deazariboflavin cofactor are not interconverted by *S. lincolnensis*, the mutation in strain NTG-3 must be located before a branch point in the biosynthesis of the two compounds. LE VAN *et al.*[7] have recently proposed a pathway for the biosynthesis of 7,8-didemethyl-8-hydroxy-5-deazariboflavin in *Methanobacterium thermoautotrophicum*. Riboflavin and the deazariboflavin are postulated to share a common intermediate, 5-amino-6-(ribitylamino)-2,4(1*H*,3*H*)-pyrimidinedione 5′-phosphate, in a biosynthetic pathway starting from GTP. If this pathway also occurs in streptomycetes, the NTG-3 mutation must block a biosynthetic step in the conversion of GTP to the pyrimidinedione intermediate.

Studies by BRAHME *et al.* have focused on the lincomycin A biosynthetic pathway in *S. lincolnensis*.[8,9] It was postulated that a synthetase condenses propylproline (PPL) and methylthiolincosaminide (MTL) to produce *N*-demethyllincomycin A. A methyl transferase then converts *N*-demethyllincomycin A to lincomycin A. The biosynthesis of MTL is postulated to proceed from glucose through an octulose. In experiments using deuterated amino acids and carbon-13 enriched glucose, the synthesis of PPL was shown to proceed from tyrosine through L-DOPA to a precursor of PPL.[8]

To test whether the LCF is involved in the biosynthesis of MTL or in the biosynthesis of PPL, the two compounds were added at concentrations of 200 μg/ml to standard NTG-3 assay fermentations. The results, shown in Table 1, clearly indicate that the cofactor must be acting at an enzymatic step leading to the synthesis of propyl proline and that the lincomycin synthetase and the methyl transferase are functional in mutant NTG-3.

Since tyrosine and L-DOPA have been shown by isotope incorporation experiments to be precursors of PPL,[8] they were also added to NTG-3 assay fermentations at 200 μg/ml concentrations and lincomycin production measured. Neither addition resulted in lincomycin production by mutant NTG-3. These experiments thus narrow the site of LCF action to an enzymatic step between L-DOPA and PPL in the lincomycin biosynthetic pathway.

Acknowledgments

We would like to thank Drs. J. J. MANIS, H. J. WOLF, and M. G. WOVCHA, Bioprocess R & D, The Upjohn Company, for helpful discussions and assistance. We also acknowledge the contributions of Dr. C. MEYER and W. A. MURPHY to this project.

References

1) MASON, D. J.; A. DIETZ & C. DEBOER: Lincomycin, a new antibiotic. I. Discovery and biological properties. Antimicrob. Agents Chemother.-1962: 554~559, 1963
2) COATS, J. H. & J. ROESER: Genetic recombination in *Streptomyces bikiniensis* var. *zorbonensis*. J. Bacteriol. 105: 880~885, 1971
3) HANKA, L. J.; D. J. MASON, M. R. BURCH & R. W. TREICK: Lincomycin, a new antibiotic. III. Microbiological assay. Antimicrob. Agents Chemother.-1962: 565~569, 1963
4) KERN, R.; P. J. KELLER, G. SCHMIDT & A. BACHER: Isolation and structural identification of a chromophoric coenzyme F_{420} fragment from culture fluid of *Methanobacterium thermoautotrophicum*. Arch. Microbiol. 136: 191~193, 1983
5) MCCORMICK, J. R. D. & G. O. MORTON: Identity of cosynthetic factor 1 of *Streptomyces aureofaciens* and fragment FO from coenzyme F420 of *Methanobacterium* species. J. Am. Chem. Soc. 104: 4014~4015, 1982
6) EKER, A. P. M.; A. POL, P. VAN DER MEYDEN & G. D. VOGELS: Purification and properties of 8-hydroxy-5-deazaflavin derivatives from *Streptomyces griseus*. FEMS Microbiol. Lett. 8: 161~166, 1980
7) LE VAN, Q.; B. SCHWARZKOPF, A. BACHER, P. J. KELLER, S. LEE & H. G. FLOSS: Biosynthesis of 7,8-didemethyl-8-hydroxy-5-deazariboflavin, the chromophoric moiety of coenzyme F_{420}. J. Am. Chem. Soc. 107: 8300~8301, 1985
8) BRAHME, N. M.; J. E. GONZALEZ, J. P. ROLLS, E. J. HESSLER, S. MIZSAK & L. H. HURLEY:

Biosynthesis of the lincomycins. 1. Studies using stable isotopes on the biosynthesis of the propyl- and ethyl-L-hygric acid moieties of lincomycins A and B. J. Am. Chem. Soc. 106: 7873~7878, 1984

9) BRAHME, N. M.; J. E. GONZALEZ, S. MIZSAK, J. R. ROLLS, E. J. HESSLER & L. H. HURLEY: Biosynthesis of the lincomycins. 2. Studies using stable isotopes on the biosynthesis of methylthiolincosaminide moiety of lincomycin A. J. Am. Chem. Soc. 106: 7878~7883, 1984