Journal of Microbiology and Biotechnology
Vol. 7. No. 5, 363-366, 1997

NOTE

Characterization of Doxorubicin-nonproducing Mutant, Nu3 of *Streptomyces peucetius* ATCC27952

HWANG, CHEOL KYU, HONG SUB LEE[1], YOUNG-SOO HONG, NAM SOO PAEK[1], TAE HAN KIM[1], AND JUNG JOON LEE*

[1]Research laboratories, IlDong Pharmaceutical Co., LTD., Korea
Natural Products Biosynthesis R.U., Korea Research Institute of Bioscience and Biotechnology (KRIBB), P.O.Box 115, Yusong, Taejon 305-600, Korea

A doxorubicin-nonproducing mutant, Nu23 was selected from the mutagenesis of *Streptomyces peucetius* ATCC27952. Chemical and molecular biological analysis suggested that the mutant was blocked at the step between polyketide synthase and aklaviketon reductase in the biosynthesis of doxorubicin. Furthermore, the bioconversion experiment with the mutant revealed that 13-dihydrodaunorubicin is likely to be a biosynthetic intermediate.

Complementation of block mutants in the biosynthesis of antibiotics is a common method to identify specific antibiotic biosynthesis genes (4, 16). Block mutants are also useful in determining biosynthetic intermediates by feeding the intermediate or its analogues followed by analysis of the final product. In a study of the biosynthesis of the anthracycline antibiotic, doxorubicin, we selected several mutants of *Streptomyces peucetius* ATCC27952 using UV light and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) treatment of spores. Among them a blocked mutant strain Nu23, which was unable to produce not only doxorubicin (dxr) but also daunorubicin (dnr), ε-rhodomycinone (ε-rho) and aklavinone (akn), intermediates in the biosynthesis of dxr. However, the mutant was able to convert intermediates such as ε-rho, akn, dnr, and 13-dihydrodaunorubicin (13-dhdnr) to dxr efficiently, indicating that the mutant was useful for the study of anthracycline biosynthesis. Here we report regarding the selection method and some of the characteristics of the mutant strain Nu23.

Doxorubicin, one of the most useful antibiotics in antitumor chemotherapy, is produced by *S. peucetius* ATCC 27952 (1, 18). Many research groups have studied the biosynthesis of this class of antibiotics (3, 5, 6, 14, 19). As results most of the biosynthetic intermediates have been isolated and their roles in the biosynthesis of anthracyclines have been clarified. In addition, a gene cluster encompassing all the dxr biosynthesis genes has been cloned and the function of many genes including doxorubicin resistance genes, regulatory genes, the aklavinone 11-hydroxylase gene and the daunorubicin 14-hydroxylase gene etc. have been elucidated (8, 10, 11, 15, 17). However, most of the mutants, apart from those reported by Oki and coworkers (14) and N. Crespi-Perellino *et al.* (6), were not very useful for bioconversion assay.

In the course of mutant selection, a spore suspension of *S. peucetius* ATCC27952 (2×10^7 spores in 1 ml of sterile water) was treated with NTG at a concentration of 1 mg/ml in 0.05 M of TM buffer (0.05 M Tris and 0.05 M maleic acid, pH 8.0) for 30 min. This gave 0.01% survival rates and spores were transferred to NDYE medium (4.5% maltose, 0.5% yeast extract, 0.48% HEPES, 0.43% $NaNO_3$, 0.023% K_2HPO_4, 0.012% $MgSO_4$, 0.2% 10X trace element solution [pH 7.4]) and incubated at 30°C overnight (7). The spores that germinated were spread on R2YE plates containing 100 μg/ml of dxr and incubated at 28°C for 7 days (12). Strain Nu11 was selected for its high resistance to dxr. Interestingly this mutant produced 3 fold more dnr and 10 fold more dxr than the parent strain did (data not shown). This result suggests that resistance to its own antibiotic would be an efficient selection marker for strain improvement. The strain Nu11 (2×10^6 spores in 0.1 ml of sterile water) was further treated with a UV light (15 W) at a distance of 20 cm for 1 min and the spores were spread on R2YE plates. One color mutant, Nu23 was selected out of 2500 survivors. The mutant strain Nu23 was cultivated in NDYE medium and its metabolites in the culture broth were analyzed by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) as re-

*Corresponding author
Phone: 82-42-860-4360. Fax: 82-42-860-4595.
E-mail: jjlee@kribb4680.kribb.re.kr
Key words: *Streptomyces peucetius*, mutant, bioconversion

Table 1. Bioconversion percentage of various anthracyclines by a mutant strain Nu23 derived from *Streptomyces peucetius*.

Initial chemical species	Conc. (μg/ml)[a]	*In vivo* cell bioconversion			
		ε-rhodomycinone	daunorubicin	13-dihydro daunorubicin	doxorubicin
aklavinone**	10	5%	25%	30%	35%
ε-rhodomycinone	10	9%*	20%	22%	49%
13-dihydrodaunorubicin	10	–	25%	41%*	34%
daunorubicin	10	–	45%*	39%	16%

[a]Concentration of substrates added to 3 days old culture grown in NDYE medium. *Remaining percentage of substrates after 72 h bioconversion experiment. **Aklavinone was remained less than 5% after bioconversion experiment.

ported previously (11). We found that the strain Nu23 was unable to produce dxr or other known intermediates such as akn, ε-rho and dnr, but it did produced a number of brownish and purple pigments that have not yet been identified by their structure.

We next examined the bioconversion activity of the mutant. The strain Nu23 grown in NDYE medium was transferred to a flask containing the same medium and akn (10 μg/ml), ε-rho (10 μg/ml) and dnr (10 μg/ml) was added respectively. After incubation at 30°C for 2 days the broth was extracted with chloroform:methanol (9 : 1) mixture and analyzed according to bioconversion product by HPLC. As shown in Table 1, akn and ε-rho were efficiently converted to dxr despite long biosynthetic pathway but dnr and 13-dhdnr were converted to dxr less efficiently. Daunomycinone was not converted (data not shown). The low bioconversion of dnr and 13-dhdnr may indicate that the permeability of glycoside

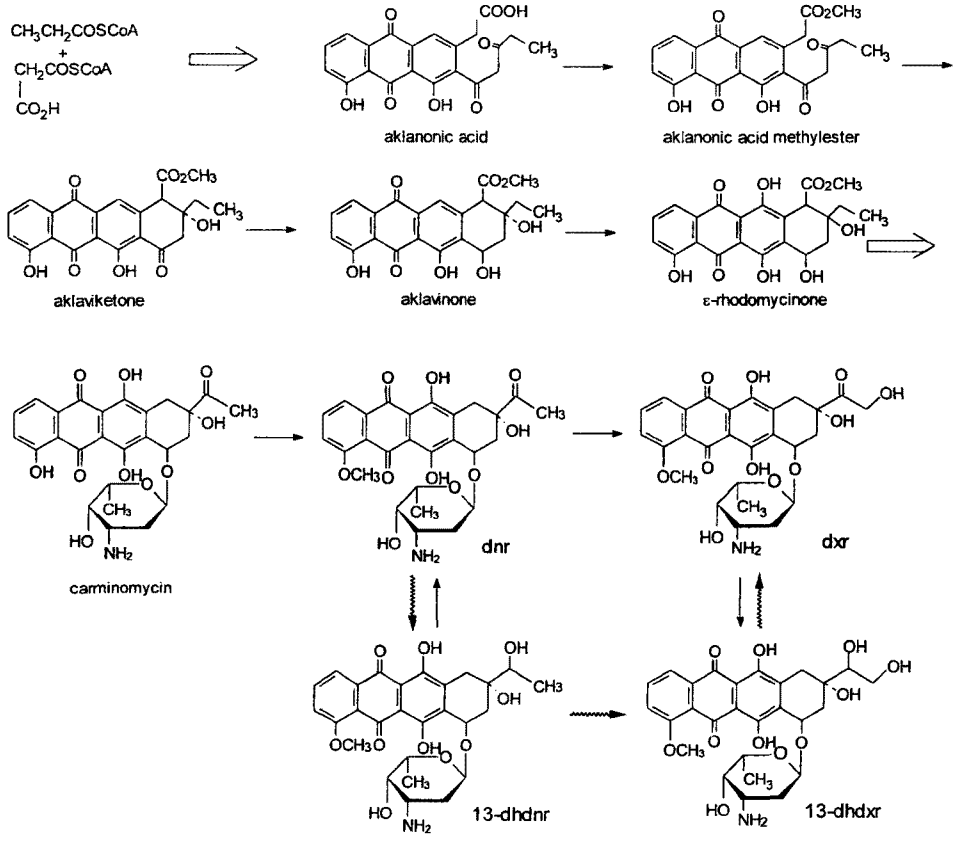

Fig. 1. Doxorubicin biosynthesis pathway.
Thick open arrows indicate that several enzymatic steps are necessary for the conversion. Thin arrow indicate a single enzymatic reaction. Wry arrow indicate a proposed biosynthetic pathway from dnr to dxr.

was not as efficient as that of aglycone. This result indicats that the strain Nu23 is probably blocked at the step between polyketide synthase and aklaviketon reductase in the biosynthesis of dxr as shown in Fig. 1. The mutant still conferred resistance to dxr and showed no complementation with dnrR2 which are positive regulatory genes in the biosynthesis of dxr (18), indicating that the mutant is different from the one reported by Ho and Chey (9).

The fact that Nu23 was unable to make ε-rhodomycinone but could convert it to doxorubicin may suggest that it was blocked in the early biosynthetic pathway i. e., polyketide synthase reaction. To test this possibility we introduced the minimal *tcm*PKS gene (*tcm*KLMN) to the mutant but no complementation on the production of anthracycline was detected (13). But, in a similar experiment with *act* PKS (*act* III, I, VII, V), the transformant produced a new yellow compound. We analysed a chloroform extract of the culture of Nu23 transformed with *act* PKS, grown for 5 days in NDYE medium and purified the compound. ^{1}H- and ^{13}C- NMR and MS spectral data were in a good agreement with those of aloesaponarin II which had also been previously identified from a *S. coelicolor* mutant blocking in actionrhodin biosynthesis and from *S. galilaeus* ATCC31133 transformed with a plasmid containing *act* PKS genes (2). On the basis of those results, a type II polyketide synthase, multienzyme system with a strong conservation of the gene and function for the constituent proteins, would not complement heterologously. Another possibility could be that the mutant might be blocked in between the step after polyketide synthase and the step prior to aklavinone, but we could not clarify this step of mutation in the biosynthesis of doxorubicin because we could not feed the early intermediates such as aklanonic acid, aklanonic acid methyl ester and aklaviketone.

In the biosynthesis of dxr, 13-dhdnr is produced large quantities by the strain *S. peucetius* ATCC27952. There were contradictory reports on the role of the compound in the biosynthesis of dxr. *Yoshimoto* and coworkers (19) reported that this compound is an intermediate but *Strohl* (3, 5) and the *Farmitalia* group (6) suggest that the compound is a shunt product. Therefore, we fed 13-dhdnr to the culture of Nu23 growing in NDYE overnight and the product was analyzed by TLC and HPLC. The result was that 13-dhdnr was transformed to dxr more efficiently than dnr was, indicating that 13-dhdnr is likely to be a immediate precursor of doxorubicin. This result can not tell us whether the biosynthetic sequence is dnr → 13-dhdnr → 13-dhdxr → dxr or 13-dhdnr → dnr → dxr, however it does suggest that 13-dhdnr is likely to be a biosynthetic intermediate. This hypothesis should be further conformed with the purified daunorubicin 14-hydroxylase.

Acknowlegment

This work was partially supported by a grant from the Korean ministry of science and technology.

REFERENCES

1. Arcamone, F. 1984. Antitumor anthracycline: recent developments. *Med. Res. Rev.* **4**: 153-188.
2. Bartel, P. L., C. B. Zhu, J. S. Lampel, D. C. Dosch, N. C. Connors, W. R. Strohl, J. M. Beale, and H. G. Floss. 1990. Biosynthesis of anthraquinones by interspecies cloning of actinorhodin biosynthesis genes in streptomycetes: clarification of actinorhodin gene functions. *J. Bacteriol.* **172**: 4816-4826.
3. Bartel, P. L. N. C. Connors, and W. R. Strohl. 1990. Biosynthesis of anthracyclines: analysis of mutants of *Streptomyces* sp. strain C5 blocked in daunomycin biosynthesis. *J. Gen. Microbiol.* **136**: 1877-1886.
4. Colombo, A. L., M. M. Solinas, G. Perini, G. Biamonti, G. Zanella, M. Caruso, F. Torti, S. Filippini, A., Inventi-Solari, and L. Garofano. 1992. Expression of doxorubicin-daunorubicin resistance genes in different anthracycline-producing mutants of *Streptomyces peucetius. J. Bacteriol.* **174**: 1641-1646.
5. Conners, N. C., P. L. Bartel, and W. R. Strohl. 1990. Biosynthesis of anthracyclines: carminomycin 4-O-methyltransferase, the terminal enzymic step in the formation of daunomycin. *J. Gen. Microbiol.* **136**: 1895-1898.
6. Crespi-Perellino, N., A. Grein, S. Merli, A. Minghetti, and C. Spalla. 1982. Biosynthetic relationships among daunorubicin, doxorubicin and 13-dihydrodaunorubicin in *Streptomyces peucetius. Experientia* **38**: 1455-1456.
7. Dekleva, M. L., J. A. Titus, and W. R. Strohl. 1985. Nutrient effects on anthracycline production by *Streptomyces peucetius* in a defined medium. *Can. J. Microbiol.* **31**: 287-294.
8. Dickens, M. L. and W. R. Strohl. 1996. Isolation and Characterization of a gene from *Streptomyces* sp. strain C5 that confers the ability to convert daunomycin to doxorubicin on *Streptomyces lividans* TK24. *J. Bacteriol.* **178**: 3389-3395.
9. Ho, C. C. and M. L. Chye. 1985. Construction of a genetic map of chromosomal auxotrophic markers in *Streptomyces peucetius* var. *caesius. J. Gen. Appl. Microbiol.*, **31**: 231-241.
10. Hong, Y. S., C. K. Hwang, D. Y. Hwang, Y. H. Kim, and J. J. Lee. 1992. Cloning and Sequencing of a gene cluster for the resistance to Doxorubicin from *Streptomyces peucetius* subsp. caesius ATCC 27952. *J. Microbiol. Biotechnol.* **2**: 153-160.
11. Hong, Y. S., C. K. Hwang., S. K. Hong., Y. H. Kim, and J. J. Lee. 1994. Molecular cloning and characterization of the aklavinone 11-hydroxylase gene of *Streptomyces peucetius* subsp. *caesius* ATCC 27952. *J. Bacteriol.* **176**: 7096-7101.
12. Hopwood, D. W., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M.

Ward, and H. Schremp. 1985. *Genetic manipulation of Streptomyces. A laboratory manual.* The John Innes Foundation, Norwich, UK.
13. Kim, E.-S., K. D. Carmer, A. L. Shreve, and D. H. Sherman. 1995. Heterologous expression of an engineered biosynthetic pathway: Functional dissection of type II polyketide synthase components in Streptomyces species. *J. Bacteriol.* **177**: 1202-1207.
14. McDaniel, R., S. Ebert-Khosla, D. A. Hopwood, and C. Khosla. 1996. Rational design of aromatic polyketide natural products by recombinant assembly of enzymatic subunits. *Nature* **375**: 549-554.
15. Oki, T., Y. Takatsuki, H. Tobe, A. Yoshimoto, T. Takeuchi, and H. Umezawa. 1981. Microbial conversion of daunorubicin, carminomycin I and feudomycin A to adriamycin. *J. Antibiot.* **34**: 1229-1320.
16. Otten, S. L., K. J. Stutzman-Engwall, and C. R. Hutchinson. 1990. Cloning and expression of daunorubicin biosynthesis genes from *Streptomyces peucetius* and *peucetius* subsp. *caesius. J. Bacteriol.* **172**: 3427-3434.
17. Stutzman-Engwall, K. J. and C. R. Hutchison. 1989. Mutigene families for anthracycline antibiotic production in *Streptomyces peucetius. Proc. Natl. Acad. Sci. USA* **86**: 3135-3139.
18. Stutzman-Engwall, K. J., S. L. Otten, and C. R. Hutchison. 1992. Regulation of secondary metabolism in *Streptomyces* spp. and overproduction of daunorubicin in *Streptomyces peucetius. J. Bacteriol.* **174**: 144-154.
19. White, R. J. and R. M. Stroshane. 1984. Daunorubicin and adriamycin: properties, biosynthesis and fermentation, p. 569-594. *In* E. J. Vandamme(ed.), *Biotechnology of industrial antibiotics*, vol. 22. Marcel Dekker, Inc., New York.
20. Yoshimoto, A., T. Nakamura, K. Kubo, O. Johdo, and H. Tone. 1995. Daunomycin biosynthesis by microbial conversion of precursor metabolites using biosynthetically blocked mutants. *J. Fermen. Bioengin.* **79**: 229-235.

(Received April 28, 1997)