Analysis of doxorubicin biosynthetic gene cluster and intensive study of regulatory system of *Streptomyces peucetious* ATCC 27952

[1]Niranjan Parajuli, [2]Young-Ho Moon, [3]Jin Suk Woo, [4]Byung Gee Kim [1]Hei Chan Lee, [1]Kwangkyoung Liou and [1]Jae Kyung Sohng

[1]Institute of Biomolecule Reconstruction, SunMoon University, Asan, 336-840, Korea
[2]Genotech corp. 461-6, jonmin-Dong,Yusong-Gu, Daejon,305-390, Korea
[3]GeneChem Inc. 505 Palace Officetel, 1316-4Seocho-dong, Seocho-Gu, Seoul, 137-070
[4]Institute of Molecular Biology and Genetics, Seoul National University, Seoul, Koea.

Introduction

Doxorubicin (DXR) and daunorubicin have been using as anticancer drugs since long time. DXR was first isolated in 1969 from *Streptomyces peucetious* ATCC27952, only organism reported for its production. (1) Nowadays, main attention is given to develop improved strains or processes for overproduction of doxorubicin and invention of its derivatives. One of the many approaches for the realization of this goal is the cloning and characterization of the gene(s) responsible for regulation of doxorubicin biosynthetic gene cluster.

In *S.peucetious* ATCC 27952, five nonoverlapping regions are found. Rhodomycin producer gene and two daunorubicin resistance genes and some genes for synthesis of aglycone portion of daunorubicin are present in region IV. (2). Other genes that influence the production of daunorubicin reside in the three other regions but they are not studied extensively due to lack of complete sequence of the genome. Restriction mapping of the DNA cloned from the strain 27952 indicated that the three regions are apparently identical to group I, III and IV clones of strain 27952. (3)

The production of antibiotics is regulated by variety of physiological and nutritional conditions. SARP and GARP proteins control antibiotic production. It is noticed from different literature that sometimes GARP, ã-butyrolactone receptor proteins may control SARP, streptomyces antibiotic regulatory proteins and mutant of specific auto regulator and response gene can enhance antibiotic production. Most of the actinomycetes have two-component regulatory system, which consists of response regulator protein and histidine kinase sensor.

Response regulator protein resides on pathway specific systems influencing expression in antibiotic biosynthesis gene clusters but they do not appear to be associated with a histidine kinase sensor gene. Different studies show that regulatory gene(s) may or may not be cluster with biosynthetic pathway (4). The gene dnrI is supposed to be pathway specific, positive regulatory gene, which binds specifically to DNA fragments containing promoter regions in daunorubicin gene cluster. It has high sequence homology with *act*II gene from *S.coelicolor*. Similarly, dnrO, negative regulator gene which encodes a DNA-binding protein

and regulates daunorubicin production in *S.peucetious* by controlling expression of dnrN pseudo response regulator gene

Therefore, we will pay our attention to find global regulatory genes as well as other regulating elements after analyzing the complete sequence of the genome.

Materials and Methods

Chemicals:

Endonuclease restriction enzymes were purchased from Takara Company, Japan. Packaging extract of Epicenter Company was used for construction of genomic library

Bacterial strains, Plasmids and culture conditions

E.coli XL1 Blue MRF (Stratagen)) was used for plasmid preparation. *E. coli* strains were cultured in Luria-Bertani (LB) broth or in LB agar plates **a**t 37°℃ with or without antibiotics. *E.coli* XL1 Blue and *E.coli* (ET12567) were used as the host for preparation of the disruption plasmids.

DNA manipulations

Plasmids were isolated from *E.coli* by the alkaline lysis method. Restriction digestion, ligation and other recombinant DNA techniques were carried out according to the standard protocol (5).

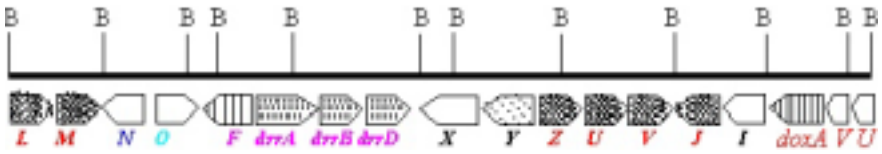
DNA Sequencing Genomic library was constructed using packaging extract of Epicenter Company with pOJ446 vector. Library was screened by colony hybridization using probe of *dnrF*, polyketide hydroxylase and *dpsY* polyketide cyclase. Sequencing was done from shotgun library and cosmid DNA, cos27-8 and cos 27-24.

Cloning and disruption of regulatory gene

The dnrI gene was amplified from the *Streptomyces peucetious* ATCC 27952 by using two synthetic oligonucleotide primers. The forward primer (SP29l-a) 5- CGG GGA TCC CAC TGT GAC-3, reverse Primer (SP29l-b) 5-GTC CCC CGG GCC GAT GCC -3. The PCR was performed in a total volume of 50 ul with 2 units of Taq-DNA polymerase, 10% dimethylsulfoxide, 2.5mM MgCl2 and 0.125 mM dNTPs in the appropriate buffer. Temperature cycling was performed by a thermocycler (Eppendorf). Pre-denaturation was carried out at 95°C for 1 min, annealing at 63℃° for 1 min and polymerization at 74°C for 1 min. The PCR product was isolated from low melting agarose, purified, digested with *Bam*HI and *Sma*I, and cloned into pEZ-T vector to make pTdnr plasmid. *Bam*HI/*Eco*RI fragment from pTdnr was taken and cloned to same sites of pWHM3 to form pWHM100. Again, SacI digested pWHM100 so that 230 bp removed from the previously cloned plasmid and 1kb neomycin resistance gene was inserted on the *Sac*I site and plasmid so formed is named as pWHM101. Similarly, pWHM102 was prepared after allowing pWHM100 digested by *Sac*I for self-ligation. The pWHM101 and pWHM102 were used as disruption plasmid to make insertional and deletion mutant of regulatory genes.

Results and discussion

The nucleotide sequences were analyzed by using DNA strider DNA and using the codon bias of Streptomycin DNA and BLAST programs on the National Center for Biotechnology Information server performed protein sequence homology search of databases. Multiple sequence alignments were performed using computer-aided database, clustalW. We found many genes of primary and secondary metabolites.

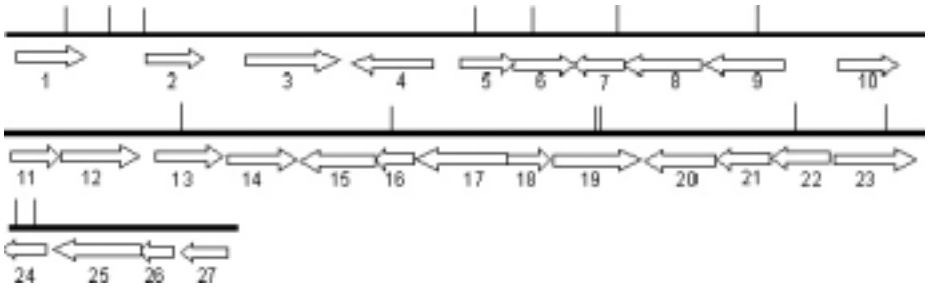


orf 10, dnrU	*daunorubicin C-13 ketoreductase*	*orf 19, dnrX*	*DNR/DOX biosynthesis gene*
orf 11, dnrV	*doxorubicin biosybthesis gene*	*orf 20, drrD*	*resistance gene*
orf 12, doxA	*C-13 oxidation,C-14 hydroxylase*	*orf 21, drrB*	*resistance gene*
orf 13, dnrI	*positive regulator*	*orf 22,drrA*	*resistance gene*
orf 14, dnmJ	*C-3 aminotransferase*	*orf 23,dnrF*	*aklavinine C-11 hydroxylase*
orf 15, dnmV	*TDP-4-keto-2,3,6-trideoxyhexulose reductase*	*orf 24,dnrO*	*repressor gene*
orf 16, dnmU	*putative epimerase*	*orf 25,dnrN*	*pseudo response regulator gene*
orf 17, dnmZ	*putative flavoprotein*	*orf 26,dnmM(dnrM)*	*TDP-glucose-4,6-dehydratase*
orf 18, dpsY	*polyketide cyclase (third cyclase)*	*orf 27,dnmL*	*G-1-P thymidylyltransferase*

References

Orf 10-19 (contig 25), Orf 20-27 (contig 2268)

Some other genes found in streptomyces peucetious ATCC27952 are listed below showing their identity with streptomyces peucetious ATCC29050.



ORFs	**Organism**	**Proposed function**	**% of identity**
orf4	*Streptomyces peucetius*	daunorubicin-doxorubicin polyketide synthase	94
orf5	*S. peucetius*	daunorubicin doxorubicin polyketide synthase	97
orf6	*S. peucetius*	PKS, dehydratase	95
orf7	*S. peucetius*	PKS (DnrH)	82
orf8	*S. peucetius*	putative baumycin biosynthesis protein	93
orf9	*S. peucetius*	putative baumycin biosynthesis protein	93
orf10	*S. peucetius*	daunorubicin C-13 ketoreductase	80
orf11	*S. peuceutius ATCC29050*	doxorubicin biosynthesis gene (DnrV)	94
orf12	*S. peuceutius*	daunorubicin C-14 hydroxylase	100
orf13	*S. peuceutius*	regulatory gene (DnrI)	100

orf14	*S. peuceutius*	daunorubicin biosynthesis sensory transduction protein (DnrJ)	90
orf15	*S. peuceutius*	thymidine diphospho-4-keto-2,3,6-trideoxyhexulose reductase	88
orf16	*S. peuceutius*	Putative epimerase	95
orf17	*S. griseus*	hypothetical protein	76
orf18	*S. peuceutius*	polyketide cyclase (DpsY)	88
orf19	*S. peuceutius*	daunorubicin-doxorubicin biosynthesis gene (DnrX)	89
orf20	*S. peuceutius*	anthracycline production regulatory gene (DnrW)	91
orf21	*S. peuceutius*	daunorubicin resistance transmembrane protein	97
orf22	*S. peuceutius*	daunorubicin resistance ATP binding protein(DrrA)	97
orf23	*S. peuceutius*	aklavinone C-11 hydroxylase	93
orf24	*S. peuceutius*	putative repressor	100

References

1. Natalie L., Sharee L.O., Yukiko D.K., Leonid F., Xiao C. L., Toshio T., Augusto I.S., Silvia F., Francesca T., Anna L.C., and Hutchinson R., Journal of Bacteriology, 181, No.1, p305-318 (1999).
2. Sharee L.O., Kim J.S.E., and Hutchinson R., Journal of Bacteriology 172, No.6, p3427-3434 (1990).
3. Sharee L.O., Kim J.S.E., and Hutchinson R., Journal of Bacteriology, 174, No. 1, p144-154 (1992).
4. Engel P., Scharfenstein L. L., Dyer J. M., Cary J. W., AppI Microbiol Biotechnol, 56, p414-419 (2001).
5. Sambrook, J., Fritsch, E. F. and Manitis, T. Molecular cloning: A Laboratory manual**.** Cold Spring Harbor, NY: Cold spring Harbor Laboratory (1989).