농학박사학위논문

A Study on the Heterologous Expression of Nonactin Biosynthetic Gene Cluster Ortholog in *Streptomyces albus* J1074

방선균 유래 Nonactin 생합성 상동유전자의 이종숙주발현에 관한 연구

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박 완 기

A Dissertation for the Degree of Doctor of Philosophy

A Study on the Heterologous Expression of Nonactin Biosynthetic Gene Cluster Ortholog in *Streptomyces albus* J1074

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이 논문을 농학박사학위논문으로 제출함

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2017년 7월

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A Study on the Heterologous Expression of Nonactin of Biosynthetic Gene Cluster Ortholog in *Streptomyces albus* J1074

Advisor: Ki-Bong Oh

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DOCTOR OF PHILOSOPHY

to the Faculty of

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Department of Agricultural Biotechnology

at

SEOUL NATIONAL UNIVERSITY

by

Wanki Park

Date Approved

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ABSTRACT

Marine-derived actinomycetes, *Streptomyces puniceus* MBL39 and *Streptomyces puniceus* Act1085, were used in this study to discover novel secondary metabolites. After analysis of sequence of 16S rRNA genes sequence and the morphological characteristics, these cell lines proved to be the same strains. From previous research that was isolation and characterization from the extract of the culture material of *Streptomyces puniceus* Act1085 by KIOST (Korea Institute of Ocean Science & Technology), two known nonactin derivatives, Dimeric dinactin and Cyclo-homononactic acid were found, but nonactin was not isolated. Therefore, it could expect that *Streptomyces puniceus* Act1085 would possess the shorter and similar nonactin biosynthetic gene cluster. To investigate if *Streptomyces puniceus* Act1085 possessed the expected genes for the synthesis of the nonactin derivatives, genomic library was constructed and screened. The ORFs of isolated gene cluster from *S. puniceus* Act1085 revealed high amino acid sequence identity, from 82% to 96%, to the corresponding ORFs of the nonactin biosynthetic gene cluster from *Streptomyces griseus* subsp. *griseus* ETH A7796. Unlike the expectation that nonactin or nonactin derivatives would be made from heterologous expression of the gene cluster in *Streptomyces albus* J1074, nocardamine was isolated. Because of the unexpected discovery, the correlation of between nonactin and nocardamine biosynthesis was checked out, but there were not any significant amino acid sequence homology between nocardamine and nonactin biosynthetic genes. From recently published complete sequence of the *S. albus* J1074, one siderophore biosynthetic gene cluster was found, and the gene cluster was considered a biosynthetic genes for nocardamine. Despite checking the presence of nocardamine biosynthetic genes in the *S. albus* J1074, it is widely used laboratory strains as a host for the heterologous

expression, and gene clusters for bioactive compounds in the *S. albus* J1074 is known to be silenced. Therefore, unexpected nocardamine synthesis in the *S. albus* J1074 was assumed to be due to the one part of transformed 26 kb gene cluster. The additional subcloning of parts of the cluster and its heterologous expression data indicate that the production of nocardamine in *S. albus* J1074 is due to *nonG* ortholog from *S. puniceus* Act1085.

Key words : Secondary metabolites, *Streptomyces puniceus*, Silent biosynthetic gene clusters, Nonactin, Heterologous expression, Nocardamine, *Streptomyces albus* J1074

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LIST OF ABBREVIATIONS

BGCs Biosynthetic gene clusters

NRPSs Nonribosomal peptide synthetases

PKSs Polyketide synthases

MRSA Methicillin-resistant *Staphylococcus aureus*

VREF Vancomycin-resistant *Enterococcus faecium*

TLC Thin layer chromatography

MIC Minimum inhibitory concentration

BLAST Basic local alignment search tool

RT-PCR Reverse transcription-polymerase chain reaction

PEG Polyethylene glycol

ESI-MS Electrospray ionisation mass spectrometry

FAB-MS Fast atom bombardment mass spectrometry

TFTRs TetR family transcriptional regulators

INTRODUCTION

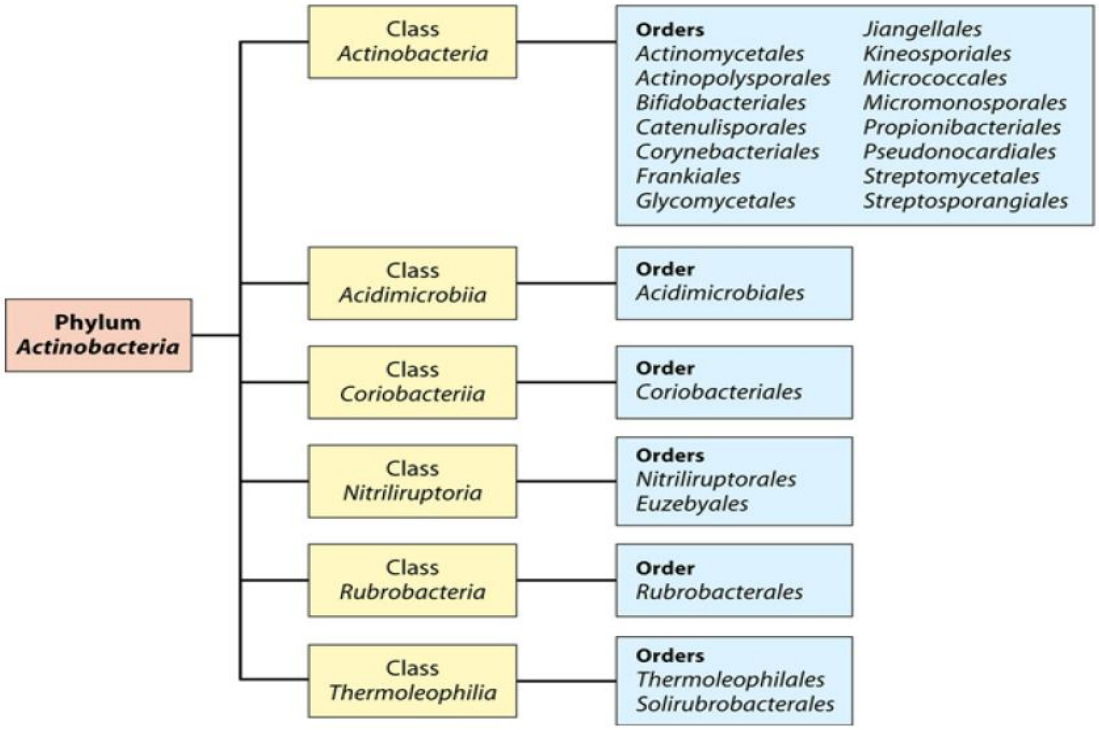
1. Actinomycetes

The phylum *Actinobacteria* represents one of the largest and most diverse phyla within the domain Bacteria (Ventura *et al*., 2007). The phylum *Actinobacteria* is Gram-positive filamentous bacteria with a high GC content generally over 55% in genomic DNA and divided into six classes, namely, *Actinobacteria*, *Acidimicrobiia*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria*, and *Thermoleophilia* (Gao and Gupta, 2012) (Figure 1). The class *Actinobacteria*, or the actinomycetes, is the largest within the phylum *Actinobacteria,* contains a total of 15 orders, namely, *Actinopolysporales*, *Catenulisporales*, *Corynebacteriales*, *Frankiales*, *Glycomycetales*, *Jiangellales*, *Kineosporiales*, *Micrococcales*, *Micromonosporales*, *Propionibacteriales*, *Pseudonocardiales*, *Streptomycetales* and *Streptosporangiales* including both previously proposed orders *Actinomycetales* and *Bifidobacteriales* (Zhi *et al*., 2009).

Figure 1. **Current taxonomic outline for the phylum *Actinobacteria*.**

(Adapted from Gao and Gupta, 2012)

The phylum *Actinobacteria,* which constitutes one of the largest phyla within the *Bacteria*, is now divided into six classes. The taxonomic ranks of subclasses and suborders are eliminated, and they are now elevated to the ranks of classes and orders, respectively.

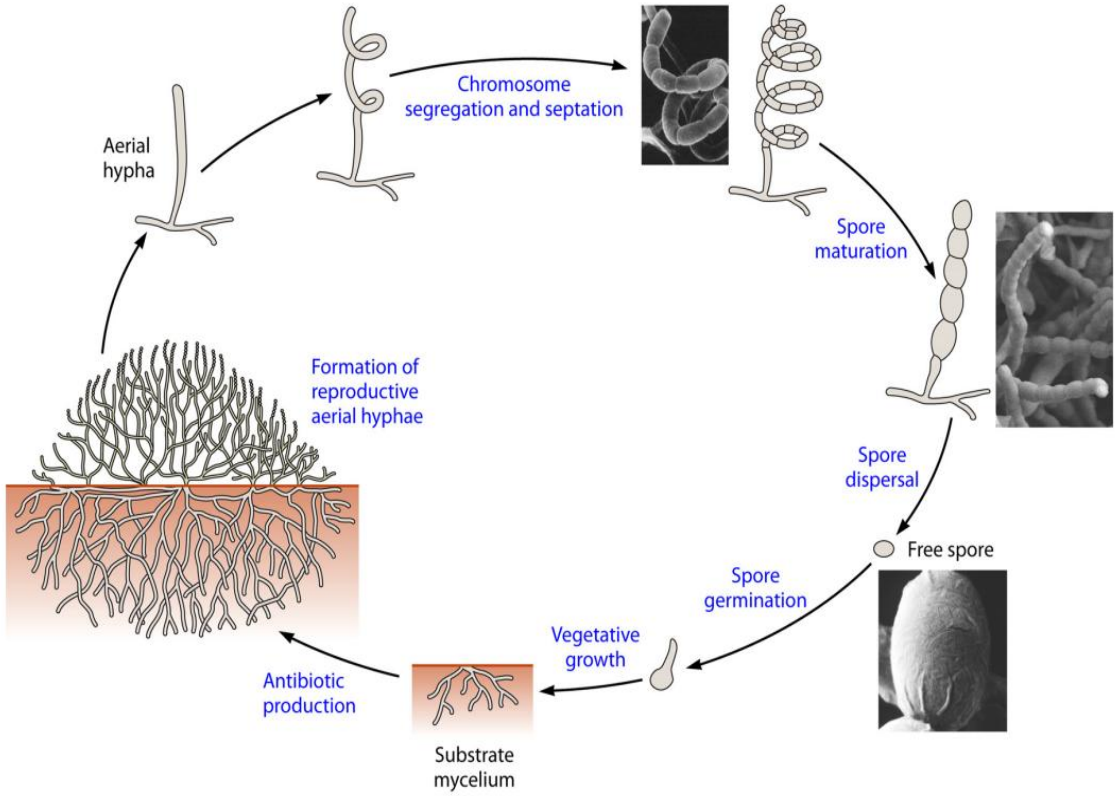

Phylum
Actinobacteria
Class
Actinobacteria
Orders
Actinomycetales
Actinopolysporales
Bifidobacteriales
Catenulisporales
Corynebacteriales
Frankiales
Glycomycetales
Jiangellales
Kineosporiales
Micrococcales
Micromonosporales
Propionibacteriales
Pseudonocardiales
Streptomycetales
Streptosporangiales
Class
Acidimicrobiia
Order
Acidimicrobiales
Class
Coriobacteriia
Order
Coriobacteriales
Class
Nitriliruptoria
Orders
Nitriliruptorales
Euzebyales
Class
Rubrobacteria
Order
Rubrobacterales
Class
Thermoleophilia
Orders
Thermoleophilales
Solirubrobacterales

Actinomycetes undergo a somewhat complex life cycle (Figure 2). Most of the *Actinobacteria* produce a mycelium, like filamentous fungi, but spend the majority of their life cycles as semidormant spores, especially under nutrient depletion which is accompanied by development of aerial hyphae, leading ultimately to sporulation (Mayfield *et al*., 1972). Therefore, actinomycetes were once considered realatives of fungi based on their ability to form mycelia which resemble those characteristics of fungi. This is what gave them their name. The name "Actinomycete" was derived from the Greek word "aktis" (a ray beam) and "mykes"(fungus) (Alexander *et al*., 2000).

Figure 2. **Schematic representation of the life cycle of sporulating actinomycetes.**

(Adapted from Barka *et al*., 2016)

Actinomycetes grow up like a combination of tip extension and branching of the hyphae. Many *Actinobacteria* produce a mycelium, like filamentous fungi, and many of these mycelial actinomycetes reproduce by sporulation.

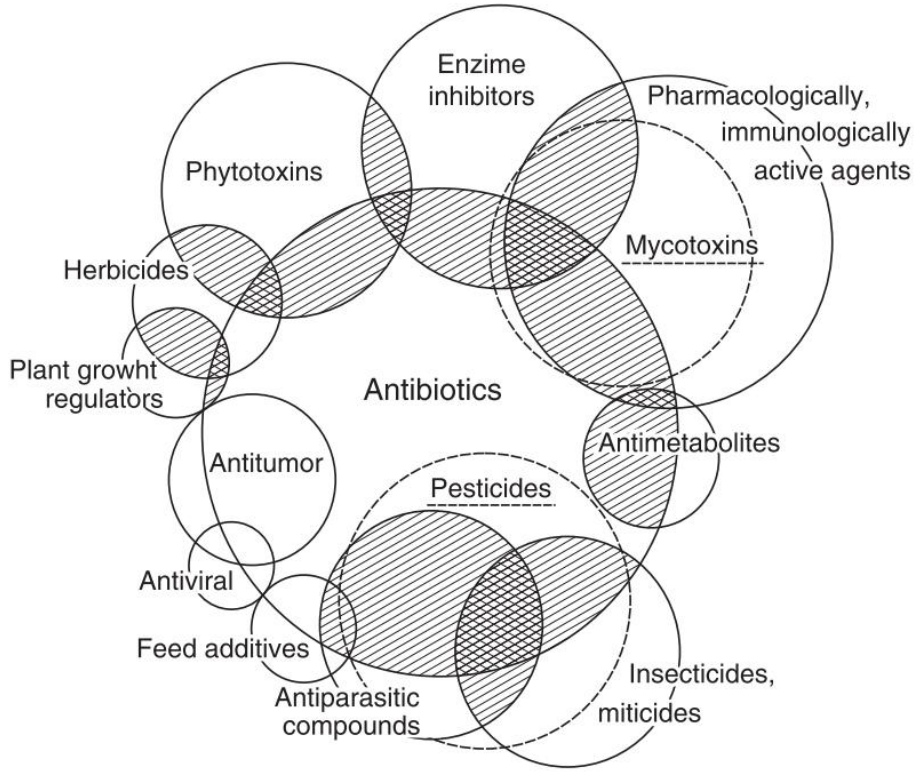

Aerial hypha
Chromosome segregation and septation
Spore maturation
Spore dispersal
Free spore
Spore germination
Vegetative growth
Antibiotic production
Substrate mycelium
Formation of reproductive aerial hyphae

These bacteria are widely distributed in nature (soil, aquatic environments, and the air) and have historically been a leading source for bioactive metabolites (Sanglier *et al*., 1993). Actinomycetes produce a vast array of compounds, also called secondary metabolites, useful as antibacterial, antifungal, antitumor and agricultural applications (as sources of insecticides, herbicide) , etc. (Bérdy, 2012) (Figure 3).

Figure 3. **Bioactive microbial metabolites.**

(Adapted from Bérdy, 2012)

Up to mid-2011, almost 30,000 different types of natural compounds with 'other' bioactivities were reported, representing more than 16,000 microbial compounds, and many of microbial metabolites possess several bioactivities. There is a high degree of overlap among activities.


Enzime
inhibitors
Pharmacologically,
immunologically
active agents
Phytotoxins
Mycotoxins
Herbicides
Plant growht
regulators
Antibiotics
Antimetabolites
Antitumor
Pesticides
Antiviral
Feed additives
Antiparasitic
compounds
Insecticides,
miticides

2. *Streptomyces*

The phylum *Actinobacteria* contains >300 genera but new taxa continue to be discovered (Euzéby, http://www.bacterio.net/). Among this group, members of the genus *Streptomyces* are the most abundant group, which differ greatly in their morphology, physiology, and biochemical activites. Interestingly, the genus *Streptomyces* are the most prolific producers of secondary metabolites such as antibiotics, antifungals, antivirals, antitumorals, anti-hypertensives, and immunosup pressants (Taddei *et al*., 2006). Among this secondary metabolites, over two-thirds of the clinically useful antibiotics are derived from *Streptomyces* sp. (Bérdy, 2012; Hopwood, 2007). The first discovery of antibiotics obtained from *Streptomyces* species was the aminoglycoside antibiotic streptomycin, discovered in the laboratory of Selman Waksman in 1943, from a *Streptomyces griseus* and used to treat tuberculosis. With the discovery of streptomycin, scientists tried to bring the search to bear on finding antibiotics within the genus, whereupon many other antimicrobial natural products have been isolated from *Streptomyces* species (Figure 4).

Figure 4. **Key findings and dates of antibiotics. Highlights of the *Streptomyces.***

(Adapted from Procópio *et al.*, 2012)

The first discovery of antibiotics obtained from *Streptomyces* species was the aminoglycoside antibiotic streptomycin, discovered in the laboratory of Selman Waksman in 1943, from a *Streptomyces griseus*. Over two-thirds of the clinically useful antibiotics are derived from *Streptomyces* sp.


2000
1970
1960
1950
1940
Platensimycin2006 S. platensis
Daptomycin2003 S. roseosporus
Linezolid2000 Synthetic
Mupirocin1985 Pseudomonas fluorescens
Ribostamycin1970 S. ribosidificus
Fosfomycin1969 S. fradiae
Trimethoprim1968 Synthetic
Gentamicin1963 Micromonospora purpurea
Fusidic acid1963 Fusidium coccineum
Nalidixic acid1962 Synthetic
Tinidazole1959 Synthetic
Kanamycin1957 S. kanamyceticus
Rifamycin1957 Amycolatopsis mediterranei
Noviobiocin1956 S. niveus
Vancomycin1956 S. orientalis
Cycloserine1955 S. garyphalus
Lincomycin1952 S. lincolnensis
Erithromycin1952 Saccharopolyspora erythraea
Virginiamycin1952 in S. pristinaespiralis S. virginiae
Isoniazid1951 Synthetic
Viomycin1951 S. vinaceus e S. capreolus
Isoniazid1951 Synthetic
Viomycin1951 S. vinaceus e S. capreolus
Nystatin1950 S. noursei
Tetracycline1950 S. aureofaciens
Neomycin1949 S. fradiae
Chloramphenicol1949 S. venezuelae
Polymyxin1947 Bacillus polymyxa
Nitrofurantoin1947 Synthetic
Cephalosporins1945 S. clavuligerus
Bacitracin1945 Bacillus licheniformis
Cephalosporins1945 S. clavuligerus
Streptomycin1944 S. griseus
Penicillin1941 Penicillium chrysogenum

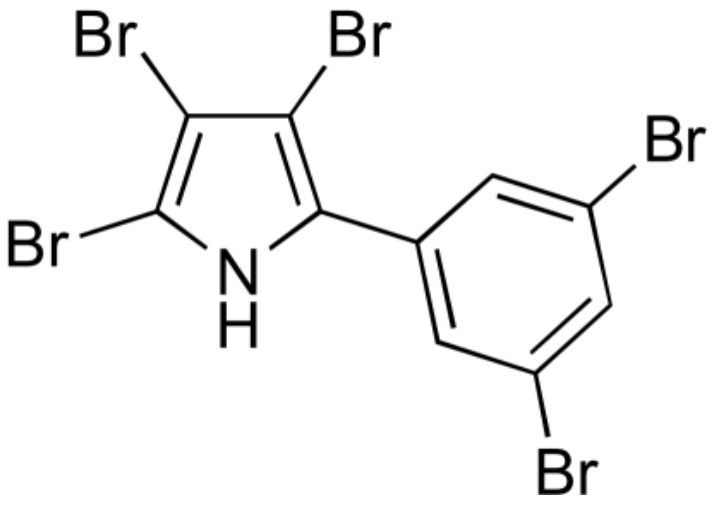
Despite the success of the discovery and production of antibiotics, more strains of pathogens have become antibiotic resistant, growing worldwide public health problem. It means that few therapeutic options remain available. Methicillin-resistant *Staphylococcus aureus* (MRSA), which is resistant not only to methicillin but usually also to aminoglycosides, macrolides, tetracycline, chloramphenicol, lincosamides, and vancomycin-resistant *Enterococci* (VRE) are particularly well-known examples (Nikaido, 2009). So, there is a great need continuously for developing new antibiotics to treat bacteria that became resistant to existing antibiotics.

3. Marine natural products

Since the discovery of penicillin in 1929, a significant amount of studies has been focused on the successful isolation of novel actinomycetes from terrestrial sources for a long time. However, the rate of discovery of new compounds from terrestrial actinomycetes has began to decrease (Lam, 2006). In this respect, mining novel sources, such as the marine environment is crucial for the isolation of new groups of actinomycetes from unexplored habitats that presumably harbors the most biodiversity and may be the vastest resource to discover novel bioactive secondary metabolites (Montaser and Luesch, 2011). The sea, covering more than 70% of the earth's surface and over 90% of the volumn of it's crust. As marine environmental conditions are massively complex and extremely different from terrestrial ones (pressure, salinity, temperature), it is surmised that marine-derived microorganisms have developed strain diversity, unique metabolic and physiological capabilities that not only ensure survival in extreme habitats, but also contribute the chemical diversity which is different types of bioactive compounts from terrestrial microorganisms (Fenical, 1993).

As expected, the physically and chemically different conditions in the marine enviroment, marine actinomycetes produce a variety of molecules with unique structural features (Doshi *et al*., 2011). The first antibiotic substance from the marine bacteria, given the name *Pseudomonas bromoutilis*, to be isolated and identified was 2-(3',5'-dibromo-2'-hydroxyphenyl)-3,4,5-tribromopyrrole, containing more than 70% by weight of bromine (Figure 5).

Figure 5. **2-(3',5'-dibromo-2'-hydroxyphenyl)-3,4,5-tribromopyrrole.**

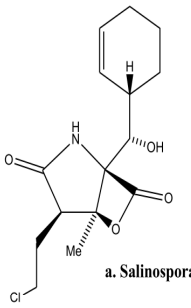

Br
Br
Br
Br
N
H
Br
Br

This pyrrole compound was shown to be highly active against Gram-positive bacteria. Strains of *Staphylococcus aureus*, *Diplococcus pneumoniae*, and *Staphylococcus pyogenes* were inhibited at an antibiotic concentration of 0.0063 μg/ml in broth culture (Trypticase Soy Broth). However, the antibiotic failed to show an activity against Gram-negative bacteria and *Candida albicans* (Burkholder *et al*., 1966).

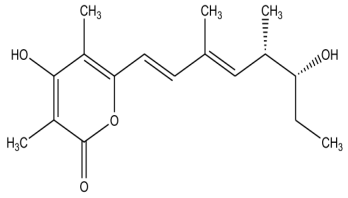
While the discovery of novel secondary metabolites from the underexploited marine-derived antinomycetes is the initial step, numerous novel metabolites possessed of unique structural features have been isolated in the past few years (Lam, 2006). Figure 6. shows some novel/new secondary metabolites produced by marine actinomycetes (Subramani and Aalbersberg, 2012).

Figure 6. **Some novel/new secondary metabolites produced by marine actinomycetes.**

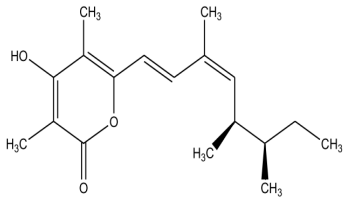
(Adapted from Subramani and Aalbersberg, 2012)



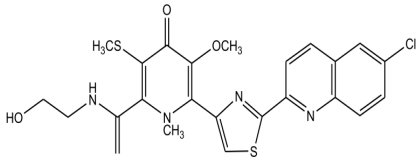
a. Salinosporamide A



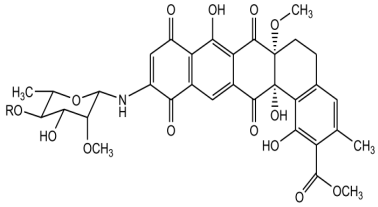
b. Salinipyrone A



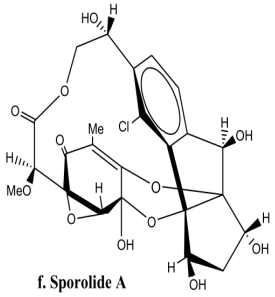
c. Salinipyrone B



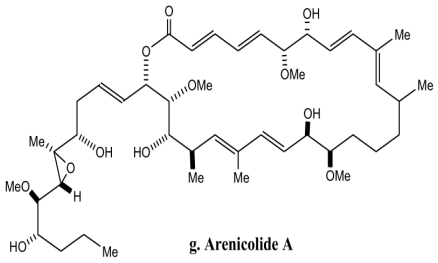
d. Lodopyridone



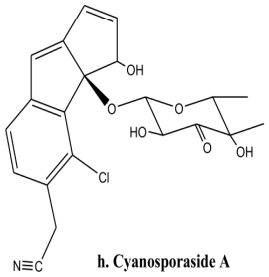
e. Arenimycin



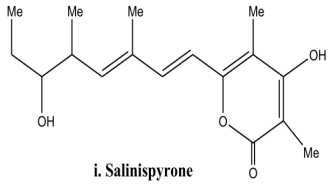
f. Sporolide A



g. Arenicolide A



h. Cyanosporaside A



i. Salinispyrone

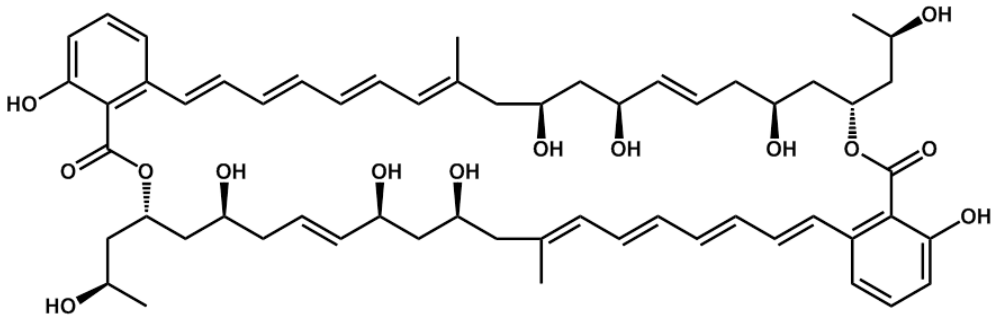
Salinosporamide A (NPI-0052) shares its fused beta-lactam-gamma-lactone bicyclic ring structure (Feling *et al*., 2003) and isolated from *Salinispora tropica* (Maldonado *et al*., 2005) which is obtained from a heat-treated marine sediment sample and requires ionic sodium for growth. Salinosporamide A (NPI-0052) is a novel proteasome inhibitor, and induces apoptosis in multiple myeloma (MM) cell lines. Commercial proteasome inhibitor anticancer drug Bortezomib has proven successful for the treatment of multiple myeloma, but resistance has already been observed in MM patients. Salinosporamide A and Bortezomib can be distinguished by chemical structure, their effects on proteasome activities, machanism of action, whereupon show synergistic anti-MM (Chauhan *et al*., 2005). This novel marine natural product from the marine actinomycete is the first clinical candidate for the treatment of a potent proteasome inhibitor used as an anticancer agent, and entered phase I human clinical trials (Fenical *et al*., 2009).

Actinomycetes are prolific producers of secondary metabolites, similarly the *Salinispora tropica* strain also produce four unprecedented compounds, Sporolide A, Arenicolide A, Cyanosporaside A and Salinispyrone A. Even though the ecological functions of *Salinispora* secondary metabolites have yet to be determined the structural diversity of these metabolites observed, which demonstrate the tremendous potential of marine actinomycetes for the production (Jensen *et al*., 2007; Subramani and Aalbersberg, 2012).

Marinomycins A-D were isolated from a new group of marine actinomycetes of the recently discovered genus *Marinospora*. Marinomycins, four antitumor-antibiotics of a new structural class, are unusual macrodiolides composed of dimeric 2-hydroxy-6-alkenyl-benzoic acid lactones with conjugated tetraene-pentahydroxy polyketide chains. The marinomycin A showed the most significant antimicrobial activities against methicillin-resistant *Staphylococcus aureus*

(MRSA) and vancomycin-resistant *Enterococcus faecium* (VREF) with minimum inhibitory concentrations (MIC_{90}) of 0.13 μM, and was impressive in selective cancer cell cytotoxicities against six of the eight melanoma cell lines in the National Cancer Institute's 60 cell lines (Kwon *et al*., 2006).

Figure 7. **Marinomycin A.**


HO
O
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HO

As described above, it is expected that marine-derived actinomycetes have a diverse specialized unique and novel secondary metabolites, because of massively complex and extremely different environment. The exploitation of metabolites from marine microorganisms is a rapidly growing field and providing opportunities to find new antibiotics to treat bacteria that became resistant to existing antibiotics.

4. Cryptic/silent biosynthetic gene clusters in actinomycetes

As noted in the last section, marine-derived anctinomycetes produce a wealth of structually unique and novel secondary metabolites with a remarkable range of biological activities. Genes encoding secondary metabolites by the actinomycete bacteria are located in clusters, so-called biosynthetic gene clusters, on the bacterial chrornosome or on plasmids, typically large (10–100 kb) and often cosists of 10-30 genes involved in biosynthesis, resistance and efflux of secondary metabolites (Martin and Liras, 1989; Bibb, 1996). However, the traditional bioactivity-based screening is a laborious process and leads too often to rediscovery of known metabolites. Because many microorganisms seem to be poorly expressed the genes to produce the of specialized ‘non-discovery’ metabolites under laboratory growth conditions (Gram, 2015).

The advent of high throughput sequencing technology is introducing sequencing of microbial genomes. Complete genome sequences of several actinomycetes and filamentous fungi have revealed that they have far greater potential to produce structurally complex, specialized metabolites than suggested by traditional approach used for bioassay-guided screening. Biosynthetic gene cluster which are present but not expressed during normal laboratory cultivation are referred to as ‘cryptic’ or ‘silent’ biosynthetic gene clusters (BGCs)

(Rutledge and Challis, 2015).

For example, *Streptomyces coelicolor* A3(2) has been studied for antibiotic production over the five decades, as a model system, and was known to make four secondary metabolites of actinorhodin (Act) (Rudd and Hopwood, 1979)**,** undecylprodigiosin (Red) (Feitelson *et al*., 1985), calcium-dependent antibiotic (CDA) (Hopwood and Wright, 1983), and methylenomycin (Mmy) (Wright and Hopwood, 1976). However, analysis of the *S. coelicolor* A3(2) complete genome sequence identified an additional 18 biosynthetic gene clusters for secondary metabolites (Bentley *et al*., 2002). Figure 8. shows that there are a lot of cryptic biosynthetic gene clusters which prevails throughout 102 actinomycetes genomes of these six, *Mycobacterium*, *Corynebacterium*, *Rhodococcus*, *Arthrobacter*, *Frankia*, and *Streptomyces* genera (Doroghazi and Metcalf, 2013).

Figure 8. **Genome size, isolation source and number of secondary metabolite gene clusters.**

(Adapted from Doroghazi and Metcalf, 2013)

The two bar plots are presented genome size (left), and number of secondary metabolite gene clusters (right). As the size of genomes increases, the number of gene clusters increases. Any compositions of cluster types found together count independently, e.g. an NRPS/PKS hybrid are counted once for NRPS and once for PKS. Actinomycetes have about 20 secondary metabolite gene clusters on average.


Number of Genes (in thousands)
10
8
6
4
2
0
Biosynthetic Gene Clusters
10
20
30
40
50
60
70
Mycobacterium africanum GM041182
Mycobacterium bovis AF2122/97
Mycobacterium tuberculosis H37Rv
Mycobacterium canettii CIPT 140010059
Mycobacterium leprae Br4923
Mycobacterium marinum M
Mycobacterium ulcerans Agy99
Mycobacterium avium 104
Mycobacterium avium subsp. paratuberculosis K-10
Mycobacterium sp. JDM601
Mycobacterium smegmatis str. MC2 155
Mycobacterium sp. Spyr1
Mycobacterium gilvum PYR-GCK
Mycobacterium sp. JLS
Mycobacterium sp. KMS
Mycobacterium sp. MCS
Mycobacterium abscessus ATCC 19977
Gordonia bronchialis DSM 43247
Tsukamurella paurometabola DSM 20162
Nocardia farcinica IFM 10152
Rhodococcus equi 103S
Rhodococcus erythropolis PR4
Rhodococcus opacus B4
Rhodococcus jostii RHA1
Corynebacterium glutamicum ATCC 13032
Corynebacterium efficiens YS-314
Corynebacterium diphtheriae NCTC13129
Corynebacterium ulcerans BR-AD22
Corynebacterium aurimucosum ATCC 700975
Corynebacterium kroppenstedtii DSM 44385
Corynebacterium variabile DSM 44702
Corynebacterium urealyticum DSM 7109
Corynebacterium resistens DSM 45100
Corynebacterium jeikeium K411
Amycolicicoccus subflavus DQS3-9A1
Segniliparus rotundus DSM 44985
Pseudonocardia dioxanivorans CB1190
Actinosynnema mirum 101T
Saccharopolyspora erythraea NRRL 2338
Saccharomonospora viridis DSM 43017
Amycolatopsis mediterranei S699
Amycolatopsis mediterranei U32
Nakamurella multipartita DSM 44233
Stackebrandtia nassauensis DSM 44728
Salinispora tropica CNB-440
Salinispora arenicola CNS-205
Verrucosispora maris AB-18-032
Micromonospora sp. L5
Micromonospora aurantiaca ATCC 27029
Geodermatophilus obscurus G-20
Frankia sp. Eul1c
Frankia sp. symbiont of Datisca glomerata
Frankia sp. EAN1pec
Frankia alni ACN14a
Frankia sp. CcI3
Acidothermus cellulolyticus 11B
Thermobispora bispora DSM 43833
Streptosporangium roseum DSM 43021
Thermomonospora curvata DSM 43183
Nocardiopsis dassonvillei subsp. dassonvillei DSM 43111
Thermobifida fusca YX
Catenulispora acidiphila DSM 44928
Streptomyces violaceusniger Tu 4113
Streptomyces bingchenggensis BCW-1
Streptomyces flavogriseus ATCC 33331
Streptomyces sp. SirexAA-E
Streptomyces griseus subsp. griseus NBRC 13350
Streptomyces avermitilis MA-4680
Streptomyces scabiei 87.22
Streptomyces coelicolor A3(2)
Kribbella flavida DSM 17836
Nocardioides sp. JS614
Microlunatus phosphovorus NM-1
Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1
Propionibacterium acnes KPA171202
Kineococcus radiotolerans SRS30216
Intrasporangium calvum 7KIP
Kytococcus sedentarius 541
Beutenbergia cavernae DSM 12333
Cellulomonas flavigena DSM 20109
Cellulomonas fimi ATCC 484
Xylanimonas cellulosilytica DSM 15894
Isoptericola variabilis 225
Sanguibacter keddieii DSM 10542
Jonesia denitrificans DSM 20603
Leifsonia xyli subsp. xyli str. CTCB07
Clavibacter michiganensis subsp. michiganensis NCPPB 382
Microbacterium testaceum StLB037
Tropheryma whipplei TW08/27
Micrococcus luteus NCTC 2665
Rothia dentocariosa ATCC 17931
Rothia mucilaginosa DY-18
Kocuria rhizophila DCC2201
Arthrobacter arilaitensis RE117
Renibacterium salmoninarum ATCC 33209
Arthrobacter aurescens TC1
Arthrobacter sp. FB24
Arthrobacter phenanthrenivorans Sphe3
Arthrobacter chlorophenolicus A6
Brachybacterium faecium DSM 4810
Arcanobacterium haemolyticum DSM 20595
Mobiluncus curtisii ATCC 43063
Bifidobacterium bifidum S17
0.2
Soil, Sludge
Pathogen
Plant-associated
Host-associated
Aquatic
Phytopathogen
Cheese
Terpene
Aminoglycoside
TOMM
Indolocarbazole
Nucleoside
Lantipeptide
Siderophore
PKS
Phosphonate
Butyrolactone
NRPS
β-lactam

High throughput sequencing technology and bioinformatics-based predictions have identified many cryptic biosynthetic gene clusters in several actinomycetes. However, a major obstacle is that these gene clusters are expressed poorly or not at all under normal laboratory fermentation conditions. When microorganisms are grown under optimized laboratory conditions, many of the environmental signals that activate regulatory systems are presumably absent, and therefore the expression of secondary metabolites BGCs is low and downregulated.

So, the development of many factors that regulate the expression of specialized secondary metabolite BGCs is recognized as important for the discovery of novel microbial natural products. The various strategies to stimulate these silent gene clusters have been successfully developed (Rutledge and Challis, 2015).

One approach to discover new bioactive compounds is the co-cultivation. Marinamides A and its methyl ester B were produced by the co-cultivation of two mangrove endophytic fungi (strains Nos. 1924 and 3893) from the South China Sea (Zhu and Lin, 2006**)**.

And, the co-culture of mycolic acid-containing bacteria *Tsukamurella pulmonis* TP-B0596 and *Streptomyces endus* S-522 generated the novel antibiotic alchivemycin A (Onaka *et al*., 2011). Another approach is the use of molecular biology. For example, manipulation of global regulators, ribsomomal engineering, and replacement of the natural promoters, so-called refactoring, with constitutive promoters have been used to activate silent biosynthetic gene clusters (Reen *et al*., 2015). *Burkholderia* Species are well-known for the human and animal pathogenic bacteria and a cryptic NRPS–PKS gene cluster was discovered. To activate this silent NRPS–PKS gene cluster, a directed promoter replacement has been used in upstream of a putative operon and succeeded in produing of burkholderic acid (Franke *et al*., 2012).

Heterologous expression, often combined with refactoring, has also been used to wake up

silent biosynthetic gene clusters. Though this application is limited to small gene clusters (<40 kb), emerging synthetic biology techniques has brought new methods to expression of an entire biosynthetic gene clusters in heterologous hosts (Rutledge and Challis, 2015). The ~34 kb pathway for production of alterochromide lipopeptides by *Pseudoalteromonas piscicida* JCM 20779 was captured using a transformation-associated recombination (TAR) strategy combined with refactoring, and expressed in the common host organism *Escherichia coli* (Ross *et al*., 2015). Polycyclic tetramate macrolactams (PTMs) contained one tetramic acid and a polycyclic system are a widely distributed class of natural products with diverse biological activities. Three novel tetramic acid-containing macrolactams was produced using synthetic biology strategy to activate a cryptic PTM biosynthetic gene cluster SGR810-815 (18,070 bp) from the sequenced *Streptomyces griseus* (Luo *et al*., 2013).

5. The purpose of this study

To discover novel secondary metabolites from the marine-derived antinomycetes, *Streptomyces puniceus* MBL39 was isolated from marine sediment which were collected surrounding mangrove plant's roots at the shore of Micronesia in December 2011, and *Streptomyces puniceus* Act1085 received from KIOST (Korea Institute of Ocean Science & Technology). *Streptomyces puniceus* Act1085 was isolated from a seafloor sediment collected in the Jeju island, Korea. After analysis of sequence of 16S rRNA genes sequence and the morphological characteristics, these cell lines proved to be the same strains. From previous work, two nonactin derivatives, Dimeric dinactin and Cyclo-homononactic acid (Figure 18), were found from the extract of the culture material of *Streptomyces puniceus* Act1085, but

nonactin and other macrotetrolides were not isolated. The present paper described that if *Streptomyces puniceus* Act1085 possessed the correlation of nonactin biosynthesis by a genomic cosmid library construction, and a discovery of unsuspected control of nocardamine production by one of transcriptional regulator in nonactin biosynthetic gene cluster for the heterologous expression host, *Streptomyces albus* J1074.

MATERIALS AND METHODS

1. Bacterial strains

Streptomyces sp. Act1085 was a gift from KIOST (Korea Institute of Ocean Science & Technology). *Streptomyces* sp. Act1085 was isolated from a seafloor sediment collected in the Jeju Island, Korea and *Streptomyces* sp. MBL39 was isolated from marine sediment which were collected surrounding mangrove plant's roots at the shore of Micronesia in December 2011. To isolate strain MBL39 from marine sediment sample, two different media were used. HV agar medium consisted of 1g humic acid dissolved in 10ml of 0.2N NaOH, 0.5g of Na_2HPO_4, 1.7g of KCl, 0.05g of $MgSO_4 \cdot 7H_2O$, 0.01g of $FeSO_4 \cdot 7H_2O$, 0.02g of $CaCO_3$, B-vitamins(0.5mg each of thiamine-HCl, riboflavin, niacin, pyridoxin-HCl, inositol, Ca-pantothenate, p-aminobenzoic acid, and 0.25mg of biotin), 22g of agar, and 1L of distilled water. AIA meidum consisted of 22g Actinomycete Isolation Agar (Himedia Laboratories, Mumbai, India), 5ml of glycerol, and 1L of distilled water. 50mg of cycloheximide was added to both of the meida afer sterilization.

Streptomyces albus J1074 (Chater and Wilde, 1980) was used as a recombinant host strain. *Escherichia coli* XL1-Blue MR supercompetent cells were purchased from Agilent Technologies.

2. Identification of *Streptomyces* sp. Act1085 and *Streptomyces* sp. MBL39

Total genomic DNA was extracted from *Streptomyces* sp. Act1085 and *Streptomyces* sp. MBL39 with i-genomic BYF DNA Extraction Mini-Kit (iNtRON Biotechnology, Korea)

according to manufacturer instruction. A conserved region of 1518 bp from 16S rRNA gene was amplified by using the primers F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R1525 (5'-AAGGAGGTGWTCCARCCGCA-3') (Lane,1991). The PCR cycles were as follows for each of the reactions detailed below : step 1= 94°C for 5min; step 2=94°C for 20s; step 3=55°C for 40s ; step 4=extension at 72°C for 1min 40s. Steps 2–4 were repeated 32 times and reaction stored at 4°C. Amplified 16S rRNA gene was cleaned by using MEGAquick-spin Total Fragment DNA Purification Kit (iNtRON Biotechnology, Korea) and cloned into pGEM-T Easy Vector System I (Promega Corp., Madison, WI, USA) according to manufacturer instruction. After sequence analysis of 16S rRNA genes of *S.* sp. Act1085 and *S.* sp. MBL39, they were matched each other. As a result, two amplified sequence of 16S rRNA genes equaled. The morphological characteristics, which produced red-purple substrate mycelium of two cell lines also would be just the same.

The amplified 16S rRNA gene sequence was matched with other sequences in the nucleotide database using the BLAST algorithm with default parameters wihin NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify related 16S rRNA sequences. To generate phylogenetic tree, the amplified 16S rRNA gene sequence of *Streptomyces* sp. Act1085 and related 16S rDNA sequences from BLAST search were performed a multiple alignment by ClustalX 2.1 (Thompson *et al*., 1997) and edited by BioEdit 7.2.5 (Hall, 1999). To construct a phylogenetic tree from the aligned sequences, MEGA 5.2 program was used (Kumar *et al*., 2001). MEGA (Molecular Evolutionary Genetics Analysis) serves many different methods. Here neighbor-joining method was utilized.

3. Culture conditions for *Streptomyces* sp. Act1085 (=MBL39) and *Streptomyces albus* J1074

Streptomyces sp. Act1085 (=MBL39) and *S.* a*lbus* J1074 was propagated Bennett's medium at 30°C for spore preparation and YMG medium (yeast extract 4g/L, malt extract 10g/L, glucose 4g/L) supplemented with 0.5% (w/v) glycine was used for genomic DNA preparation.

4. TLC-bioautography overlay assay

TLC-bioautography is used to detect antimicrobial activity in a mixture of compounds. This screening method is simple, cheap and time-saving measurement providing a "yes/no" response (Choma and Grzelak, 2011).

Streptomyces sp. Act1085 was cultured in 100ml of AP media (actinorhodin production medium, glycerol 50, glutamic acid 5g, MOPS 21g, $MgSO_4$ $7H_20$ 100mg, NaCl 100mg, KH_2PO_4 82mg, $FeSO_4$ $7H_20$ 9mg, trace element solution 2ml/L. p.H 6.5; Xiaowen Liao, *et al.*, 1995) at 28°C with shaking at 120 rpm for 10 days. The fermented culture was centrifuged at 1200g for 10 min and filtered by filter paper to seperate the mycelia. The filtrate was extracted with same volumn of ethyl acetate. The ethyl acetate extract was colleted, dried, and resuspended in MeOH. The ethyl acetate extract was applied to the silica gel TLC plate(1 cm × 10 cm) as the stationary phase. The TLC plates were developed in glass chamber containing mixture of dichloromethane and methanol (9.5 : 0.5 v/v) as the mobile phase. *Staphylococcus aureus* ATCC 25923 was incubated in 5ml LB liquid media at 37°C for 12 hours. The culture media of 200 μl were spreaded on the LB agar plate. After drying, the developed plate was faced down onto the inoculated LB agar plate and incubated at 37°C for 12 hours. And then the

zones of inhibition which formed both sides of TLC plate on the agar surface were observed.

5. Genomic DNA isolation

Chromosomal DNA isolation of *Streptomyces* sp. Act1085 (=MBL39) was performed by a previously described protocol (Nikodinovic *et al*.,2003). Genomic DNAs were prepared from mycelia from 30ml of YMG medium supplemented with 0.5% (w/v) glycine. Mycelia were haversted by centrifugation (5 min, 4000 x g), washed twice with 10.3% sucrose, and resuspended in 10ml of lysis solution (0.3M sucrose , 25ml EDTA, 25mM Tris-HCl, pH 7.5, containing 2U of RNase) in a 50ml tube. Lysozyme (10mg) and achromopeptidase (5mg; Sigma, St. Louis, MO, USA) were added as crystalline solids to the bacterial suspension and incubated at 37°C for 20 min. 10% (w/v) SDS (1ml) and proteinase K was added to a concentration of 0.5 mg/ml and the mixture was incubated at 55°C for 1.5 h. After addition of 5M NaCl (3.6ml) and chloroform (15ml), the mixture was rotated end-over-end for 20 min at 6 rpm. After centrifugation (20 min, 5000× g), the aqueous phase was transferred with wide bore pipet into a clean tube. Genomic DNA was precipitated by addition of 1 volume of isopropanol or 2 volume of ethanol and spooled using a Pasteur pipet. The spoold DNA was rinsed with 10ml 70% (v/v) ethanol to remove the mucus. The air-dried DNA was dissolved in a minimal volume of prewarmed buffer containing 10mM Tris-HCl, pH 7.4, and 10mM EDTA at 55°C.

6. Partial digestion and size fractionation of *Streptomyces* sp. Act1085 (=MBL39) genomic DNA

Total genomic DNA (100 μg) of *Streptomyces* sp. Act1085 (=MBL39) was partially digested with *Sau*3AI to give the optimal 30-45 kb fragments. The *Sau*3AI (total 1U) was added to a pre-warmed restriction enzyme reaction solution (total 600 μl), and incubated at 37°C for 10 minutes and then 0.5M EDTA added and heated at 75°C for 10 minutes to strop the enzyme reaction. The reactant was loaded on 0.3% agarose gel for 4 hours. The DNA band located between 48.5 kb (λ DNA) and 23.1 kb (λ DNA/*Hind*III) was cut off from the agarose gel and harvested by dialysis tube (SnakeSkin pleated dialysis tubing, Pierce) electrophoresis.

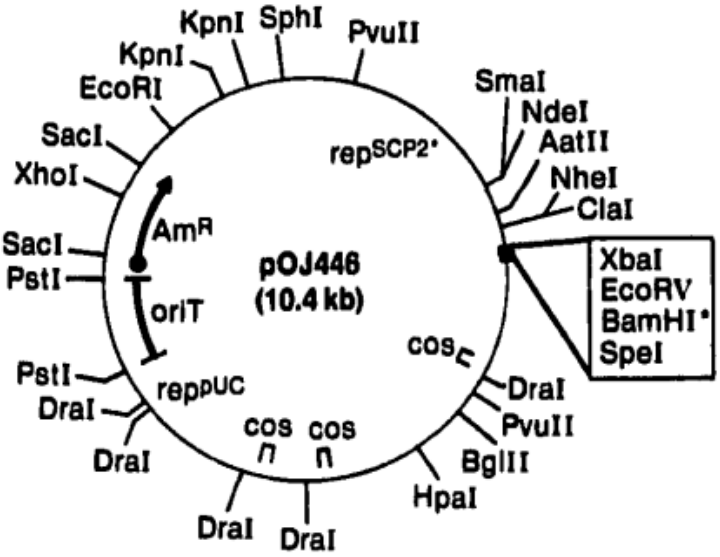
7. Digestion of the cosmid vector pOJ446 and ligation of insert genomic DNA to vector pOJ446

The pOJ446 vector was used in this cosmid genomic library construction (Bierman *et al.*, 1992) (Figure 9). The pOJ446 vector was digested with *Hpa*I/*Bam*HI, and treated calf intestinal alkaline phosphatase. The double digestion (*Hpa*I/*Bam*HI) makes two vector arms one containing one *cos*-site while the other containing two *cos*-sites which increases the efficiency of packing into phage paticles. And, dephosphorylation eliminates ligation of vector arms with each other.

After electroelution, the optimal DNA fragments (30-45 kb fragments) were ligated with 1 μg of *Bam*HI and *Hpa*I digested pOJ446 by using T4 DNA ligase.

Figure 9. **pOJ446 cosmid map.**

Plasmid pOJ446 is an apramycin-resistant *E. coli-Streptomyces* shuttle cosmid vector which can be replicated directly in *E. coli* and *Streptomyces*. It exists at low copy as a SCP2* replicon in a variety of *Streptomyces* spp. while replicating at high copy in *E. coli*.

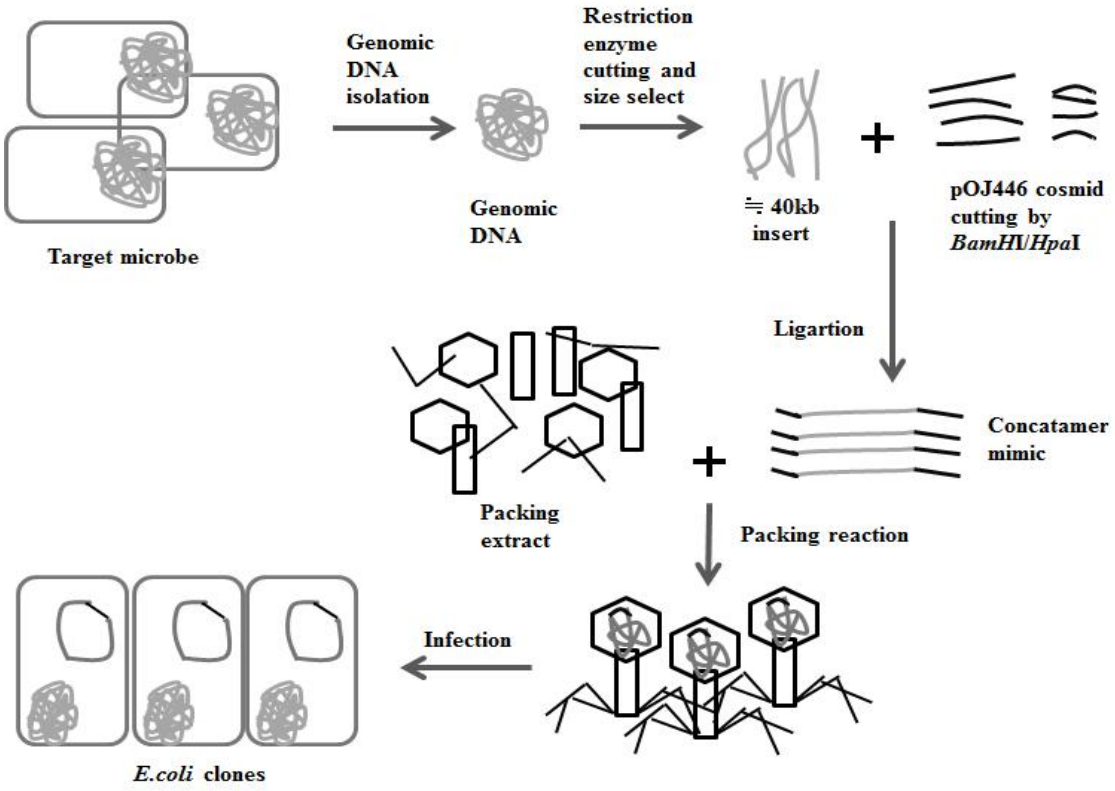

KpnI
SphI
PvuII
KpnI
EcoRI
SmaI
NdeI
AatII
NheI
ClaI
SacI
XhoI
repSCP2*
AmR
SacI
PstI
pOJ446
(10.4 kb)
orIT
XbaI
EcoRV
BamHI*
SpeI
cos
PstI
DraI
repPUC
DraI
PvuII
cos
cos
BglII
DraI
HpaI
DraI
DraI

8. *In vitro* packing reaction

The ligated cosmid arms and the partially digested genomic fragments were packaged into lambda phage heads using GigaPack III XL packaging extract (Agilent Technologies) following the protocol of the manufacturer and then used to transfect *E. coli* XL1-Blue MR supercompetent cell (Figure 10).

Figure 10. **Overview of the cosmid library construction.**

The pOJ446 cosmid vector was digested with an appropriate restriction enzymes, *Hpa*I/*Bam*HI, and dephosphorylated to provent vector self ligation. Genomic DNA fragments were generated by partial digestion of genomic DNA (100 μg) of *Streptomyces* sp. Act1085 with with *Sau*3AI to give the optimal 30-45 kb fragments. The genomic DNA fragment was ligated to the linearized vector, producing concatemer. The ligated cosmid arms and the partially digested genomic fragments were mixed with GigaPack III XL packaging extract. Molecules with two *cos* sites, approx. 35-50kb apart, would be packaged into lambda phage heads. *E. coli* XL1-Blue MR supercompetent cell was infected with the packaged phage and the recombinant cosmid was propagated as a large plasmid in the host cells. The cosmid library was screened by apramycin.


Genomic
DNA
isolation
Restriction
enzyme
cutting and
size select
Target microbe
Genomic
DNA
≒ 40kb
insert
pOJ446 cosmid
cutting by
BamHI/HpaI
Ligartion
Concatamer
mimic
Packing
extract
Packing reaction
Infection
E.coli clones

9. Library screening

A total of 1,020 cosmid-containing clones were separately picked and frozen at -80°C in 1.5ml eppendorf tubes as well as in pools of 30 clones consisting of 50 μl from each member of a 1.5ml eppendorf tube. Thus, the 1,020 individual cosmid-containing clones were also represented in 34 cosmid-containing pools. The 34 cosmid-containing pools were isolated plasmid DNA. The purified plasmid DNA from each of these 30 clones was suspended in 50 μl of TE solution (10mM Tris [pH 8.0] and 1mM EDTA) and submitted to PCR screening.

The cosmid library was screened by PCR amplification for those cosmids that contained *nonS*, nonactate synthase (Woo *et al*., 1999). The PCR cycles were as follows for each of the reactions detailed below : step 1= 98°C for 2min; step 2=98°C for 30s; step 3=67°C for 40s ; step 4=extension at 72°C for 30s. Steps 2–4 were repeated 32 times and reaction stored at 4°C. Oligonucleotides for *nonS* (5'- GACGCTCTCCCGCCATCTGCTG-3' and 5'-TGGTACGCGT CCTGGAGCCGCA-3'), which catalyzes the formation of the furan ring of nonactic acid (Walczak *et al*., 2000; GenBank accession number AF074603) were used. Nine cosmid pools (H15, H18, H19, H20, H21, H22, H27, H29, H32) revealed a positive PCR fragment of the appropriate size. Individual members of each *nonS*-positive cosmid pool were subsequently screened by PCR amplification to identify the specific cosmid containing *nonS*. Cosmid H1504 was identified in this manner. Cosmid H1504 were shotgun sequenced by Genotech (Daejeon, Korea). The H1504 clone showed to carry an insert of 35.134 kb. Open reading frames (ORFs) were deduced from sequence data by using FramePlot 3.0 (http://www0.nih.go.jp/~jun/cgi-bin/frameplot-3.0b.pl). Putative function of ORFs determined by comparison with other proteins using BLAST methods (http: //www.ncbi.nlm.nih.gov/BLAST/). Nucleotide sequence analysis of H1054 clone revealed a very high similarity (*ca.* 89%) to nonactin biosynthetic gene cluster. The

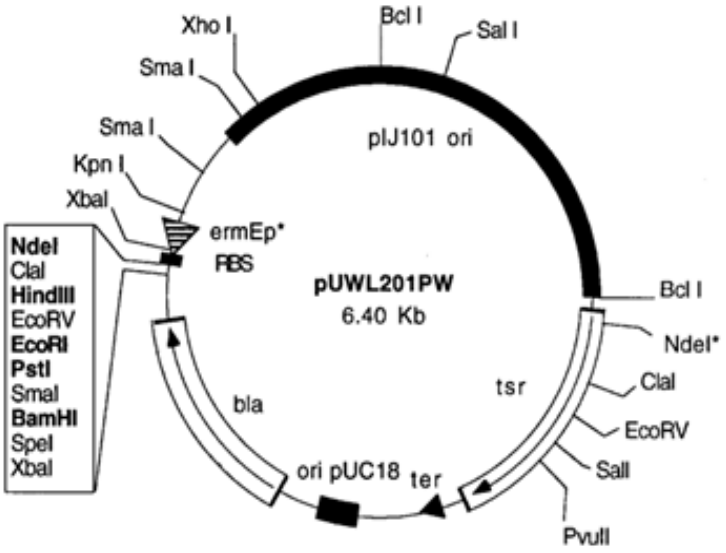
GeneBank accession numbers for the nonactin biosynthetic gene cluster are AF074603 (Walczak *et al*., 2000), AF263011 and AF263012 (Smith *et al*., 2000). The H1504 clone lacks 646 bp at its 5' end in sequence. As a results, gene cluster of H1504 clone showed a little short length as compared with nonactin biosynthetic gene cluster. The H1054 clone lacks the DNA sequence (646 bp) of the region harboring *nonX* (Kwon *et al*., 2002). Therefore, the another clone which contain similar genes of nonactin based on the H1504 sequence was found. The forward and reverse primer from *nonX* were used for library screening. The PCR cycles were as follows for each of the reactions detailed above : step 1= 98°C for 2min; step 2=98°C for 30 s; step 3=67°C for 40s ; step 4=extension at 72°C for 30s. Steps 2–4 were repeated 32 times and reaction stored at 4°C. Oligonucleotides for *nonX*-F (5'- ACCTTCTCCAGCGGCGCGGACA-3') and *nonX*-R (5'-CGCTGCAACGAAGCGCCTGCCA-3') were used (Smith *et al*., 2000; GenBank accession number AF263011). Cosmid H2129 was identified in this manner. Nucleotide sequence analysis of H2129 clone also revealed a very high similarity (*ca.* 89%) to nonactin biosynthetic gene cluster. Unfortunately, the H2129 clone lacks 775 bp at its 3' end in sequence, therefore the H2129 clone lacks the DNA sequence (775 bp) of the region harboring putative *nonH* ortholog.

10. Subcloning of 26 kb fragment

The sequence lacks of H1504 (646 bp at its 5' end) and H2129 (775 bp at its 3' end) forced us to clone the 26 kb fragment. The pUWL201PW *E. Coli*-actinomycete expression shuttle vector was used in this subcloning (Doumith *et al.*, 2000) (Figure 11) and digested with *Nde*I/*Eco*RI.

Figure 11. **pUWL201PW *E. coli*-actinomycete expression shuttle vector map.**

pUWL201PW is *Streptomyces* expression vector which has ampicillin and thiostrepton selectoin marker, and *ori* derived from pUC18 and pIJ101 for *E.coli* and S*treptomyces*, respectively. It contains the constitutive promoter *ermEp** and RBS.


Xho I
Bcl I
Sal I
Sma I
Sma I
pIJ101 ori
Kpn I
Xbal
ermEp*
RBS
NdeI
ClaI
HindIII
EcoRV
EcoRI
PstI
SmaI
BamHI
SpeI
XbaI
pUWL201PW
6.40 Kb
Bcl I
NdeI*
ClaI
tsr
bla
EcoRV
SalI
ori pUC18
ter
PvuII

For the 26 kb subcloning, PCR amplification was performed to make two 13 kb PCR fragments. The front fragment PCR was performed using H2129 cosmid clone as a template with oligonucleotides “Front 13kb-F” (5’-AGGAGAAATTACATATGACCCCTCCCGCGAAG AGCAC-3’) and “Front 13kb-R” (5’-GCACCCCTCGGAGACCAGGAAG-3’). The PCR cycles were as follows for each of the reactions detailed below : step 1= 98°C for 3min; step 2=98°C for 30 s; step 3=67°C for 30s ; step 4=extension at 72°C for 13min. Steps 2–4 were repeated 30 times and reaction stored at 4°C. The back fragment PCR was performed using H1504 cosmid clone as a template with oligonucleotides “Back 13kb-F” (5’-GTCTCCGAGGG GTGCTCGTACC-3’) and “Back 13kb-R” (5’-CGGGCTGCAGGAATTCT ACCCGCTGCCG GAGCGTAC-3’). The PCR cycles were as follows for each of the reactions detailed below : step 1= 98°C for 3min; step 2=98°C for 30 s; step 3=69°C for 30s ; step 4=extension at 72°C for 13min. Steps 2–4 were repeated 30 times and reaction stored at 4°C.

The linearized vector and the amplified two 13 kb PCR fragments were ligated and formed into one plasmid (pXH26) using In-Fusion Enzyme (TaKaRa) following the protocol of the manufacturer and then used to transform stellar competent cell (TaKaRa).

11. Total RNA isolation

Fresh cells were ground to a fine powder in liquid nitrogen and transferred to microcentrifuge tube containing TRIzol (Ambion). 200 µl of chloroform: isoamyl-alcohol (24:1) mixture were added and mixed well by vortexing. The extract was centrifuged for 20 min at 12,000 rpm and the supernatant was transferred to a new tube. Equal volume of isopropanol (IPA) was added and incubated for 10 min at -80°C. The RNA mixture was centrifuged for 20

min at 12,000 rpm. The pellet was washed with 70% cold ethanol and centrifuged for additional 5 min at 10,000 rpm. The pellet was resuspended with DEPC-treated water.

12. Reverse transcription-polymerase chain reaction

The 2μg of total RNA from each cell was reverse-transcribed by TransScript First-Strand cDNA Synthesis SuperMix using Random Primer(N9) in a 20 μl reaction mixture (Transbionovo, Beijing,China). After heat inactivation of the reaction mixture, PCR was performed using 2 μl of the first stranded cDNAs as a template with 5 pmole of primers in a 20 μl reaction. The PCR condition for *nonS* ortholog-RT was 98°C (3 min), 30 cycles of 98°C (30 sec) - 67°C (40 sec) - 72°C (30 sec), and 72°C (7 min) and for *nonT* ortholog-RT was 98°C (3 min), 30 cycles of 98°C (30 sec) - 67°C (40 sec) - 72°C (30 sec), and 72°C (7 min).

RT-PCR for a ORFs (the locus_tag XNR_4190~XNR_4187) of *S. albus* J1074 was also carried out in the same method as before. PCR products were electrophoresed on a 0.7% (w/v) agarose gel. The oligonucleotide sequences used for RT-PCR were as follows: *nonS* ortholog -RT (5'-GGGCGACGACGTGGAGAGCGTA-3' and 5'-CAGCTCCGCTCCCGCAACTCCT-3'), *nonT* ortholog -RT (5'-CACCCTCTTCCACGGCGTCGTC-3' and 5'-GGCAGCCAGCTCGTC AGCTCGA-3'), XNR_4190-RT (5'-TCAGCGTCCGCAAGTCGGCGAC-3' and 5'-GCCCATC ACCCGGAGCGTCATC-3'), XNR_4189-RT (5'-GTCACCACCGACCAGGGCGA CA-3' and 5'-ACTCGTCCCGTGCGGCGGTCTT-3'), XNR_4188-RT (5'-TGGTCCACGGCTGGGTCAC CCA-3' and 5'-TCGGGGAGCCTGCTCCTTCGTG-3'), XNR_4187-RT (5'-TCTCCGTCTCCT TCGCCGCCGA-3' and 5'-TCGAGGTAGCGGCGCAG CCACA-3').

For the reaction control, the *S.albus* 16S rRNA gene, two primers, 5'- CTTCGGTGGTG

GATTAGTGG-3' and 5'- CTTCCCTGCTGAAAGAGGTTT-3' were used.

13. Production and analysis of compound

Streptomyces albus J1074 was used as a recombinant host strain. To introduce pUWL201PW and pXH26 into *S. albus* J1074 by polyethylene glycol (PEG)-mediated methods, *Streptomyces* protoplasts were performed using standard protocols (Kieser *et al*., 2000). Spore of *S. albus* J1074 (pUWL201PW) and *S. albus* J1074 (pXH26) were cultivated in 500ml flasks containing 100ml YEME medium (yeast extract 3g/L, malt extract 3g/L, bacto-peptone 5g/L, glucose 10g/L, sucrose 340g/L) after autoclaving, supplemented with $MgCl_2 \cdot 6H_2O$ (2.5M, 2ml/L). The cultures were grown at 28°C and 220 rpm. After incubation for 7days, the cultures were harvested by centrifugation and the supernatant was extracted two times by an equal volume of chloroform and ethyl acetate. The organic layer was evaporated and dissolved in methanol and used for HPLC analysis. The analytical HPLC was conducted on Gilson Trilution LC system utilizing a reversed-phase column (Eclipse Plus C18 column, 250 x4.6nm, 5μm particle size, Agilent). A linear gradient of A (water) and B (acetonitrile) at a flow rate of 0.5ml/min was used. At first a 5 min isocratic step at 10% B, the gradient was initiated from 10 to 100% B in 25 min, and final 15 min step at 100% B. UV wavelength 210nm was used for chromatograms. To obtain purified compound, the HPLC was performed on a semi-preparative column (Eclipse XDB-C18, 250 x9.4nm, 5μm particle size, Agilent) at a flow rate of 2ml/min. The HPLC-MS measurement was recorded on LCMS-8040 (Shimadzu, Kyoto, Japan) using ESI source. From a 100ml culture, 2.8mg of nocardamine was obtained. ^{1}H and ^{13}C NMR spectra of the purified preparation were recorded on a Bruker Avance-500 (500MHz, Germany).

14. Subcloning of 16 kb fragment

For the 16 kb subcloning, PCR amplification was performed to make 3 kb PCR fragment and 13 kb PCR fragment. The 3 kb fragment PCR was performed using H1504 cosmid clone as a template with oligonucleotides "Front 3kb-F" (5'-AGGAGAAATTACATATGAGCGAGGA GTACGGCCAGGAG-3') and "Back 3kb-R" (5'-GCACCCCTCGGAGACCAGGAAG-3'). The PCR cycles were as follows for each of the reactions detailed below : step 1= 98°C for 3min; step 2=98°C for 30 s; step 3=67°C for 30s ; step 4=extension at 72°C for 3min. Steps 2–4 were repeated 30 times and reaction stored at 4°C.

The back fragment PCR was reused the "13kb back fragment" from the previous 26 kb subcloning. The linearized vector, the amplified 3 kb PCR fragment and 13 kb PCR fragment were ligated and formed into one plasmid (pXH16) using In-Fusion Enzyme (TaKaRa) following the protocol of the manufacturer and then used to transform Stellar Competent Cell (TaKaRa).

15. Subcloning of 10 kb fragment

For the 10 kb subcloning, PCR amplification was performed to make 10 kb PCR fragment. The 10 kb fragment PCR was performed using H2129 cosmid clone as a template with oligonucleotides "Front 10kb-F" (5'- AGGAGAAATTACATATGACCCCTCCCGCGAAGA GCAC-3') and "Back 10kb-R" (5'- CGGGCTGCAGGAATTCCATGCCCGTGACCAGCG CGT-3'). The PCR cycles were as follows for each of the reactions detailed below : step 1= 98°C for 3min; step 2=98°C for 30 s; step 3=67°C for 30s ; step 4=extension at 72°C for 10min. Steps 2–4 were repeated 30 times and reaction stored at 4°C. The linearized vector and

the amplified 10 kb PCR fragment were ligated and formed into one plasmid (pXH10) using In-Fusion Enzyme (TaKaRa) following the protocol of the manufacturer and then used to transform stellar competent cell (TaKaRa).

16. Subcloning of *nonB* and *nonG* ortholog

PCR amplification of *nonB* ortholog and *nonG* ortholog was performed using H1504 cosmid clone as a template. The oligonucleotide sequences were as follows: *nonB* ortholog (5'-AGGAGAAATTACATATGACTTCCGACGAGACCACGA-3') and (5'-CGGGCTGCAGGAATTCACGATCACTCCGTCGGGGTC-3'), *nonG* ortholog (5'-AGGAGAAATTACATATGAGCGAGGCACGAGGACCCGA-3') and (5'-CGGGCTGCAGGAATTCGGACAGTAAAGGCACTGAGT-3'). The PCR cycles were as follows for each of the reactions detailed below : step 1= 98°C for 3min; step 2=98°C for 30s; step 3=62°C for 30s ; step 4=extension at 72°C for 35s. Steps 2–4 were repeated 30 times and reaction stored at 4°C. The plasmid harboring *nonB* ortholog, named pXH101, and the plasmid harboring *nonG* ortholog, named pXH102 were ligated with the linearized vector using In-Fusion Enzyme (TaKaRa) following the protocol of the manufacturer and then used to transform Stellar competent cell (TaKaRa).

RESULTS

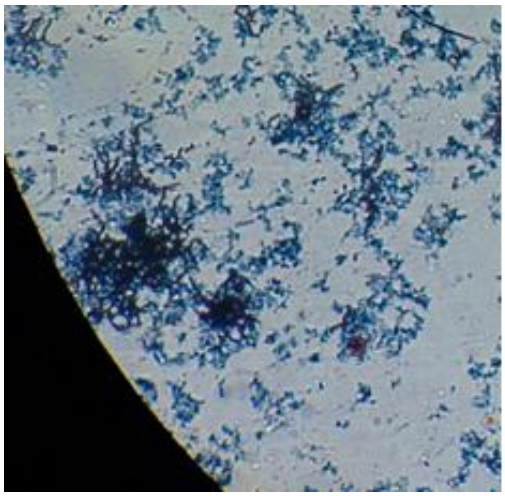
1. Strains and antimicrobial test

1.1. Strains

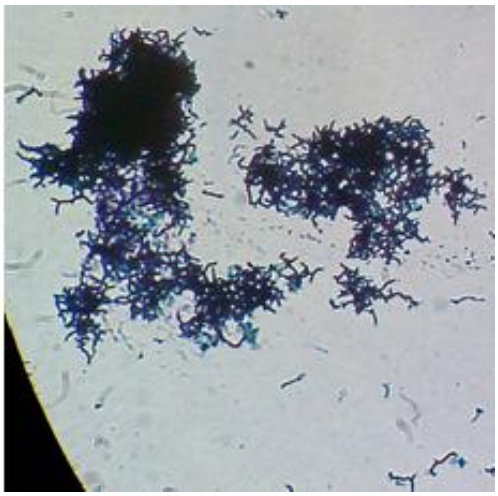
To isolate pure colony of bacteria in a mixed culture is importance, so various steps were carried out by morphological and biochemical methods. *Streptomyces* sp. MBL39 was isolated from marine sediment and *Streptomyces* sp. Act1085 was obtained from KIOST (Korea Institute of Ocean Science & Technology). Whether they were actinomycetes or not through Gram staining and morphology was checked. The primary stain Crystal violet and mordent Iodine form a strong CV-I complex all bacteria. In the case of Gram positive cells due to their thick peptidoglycan layer will retain the CV-I complex even after it is subjected to decolourization with alcohol. Hence after the gram staining, *Streptomyces* sp. MBL39 and Act1085 appeared as purple (Figure 12).

Figure 12. **Gram staining of two cell lines.**

Gram Staining is the common and most widely used staining procedure in microbiology. This test is a staining technique that differentiates the two major categories of bacteria: Gram positive and Gram negative. Two cell lines were checked whether they were actinomycetes or not through Gram staining.



Streptomyces sp. Act1085.



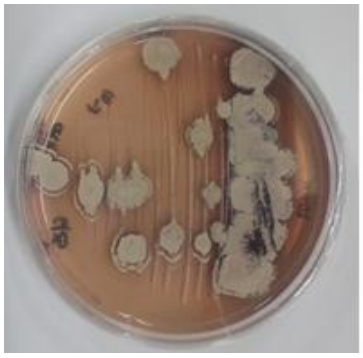
Streptomyces sp. MBL39.

Actinomycetes form filamentous mycelium and small spores, so it was determined whether two cell lines make spores or not. Through repeated streking of two cell lines, *Streptomyces* sp. Act1085 and *Streptomyces* sp. MBL39 identified the characteristics of actinomycetes (Figure 13).

Figure 13. **Morphology of two cell lines.**

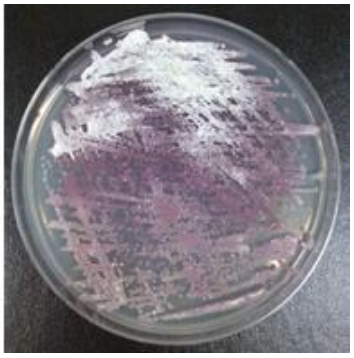
(A) *Streptomyces* sp. Act1085. (B) *Streptomyces* sp. MBL39.

(C) Process of making spores in *Streptomyces* sp. Act1085.



(A)

(B)



(C)

1.2. Antimicrobial test

The ethyl acetate extract from *Streptomyces* sp. Act1085 was conducted in TLC-bioautography overlay assay. The ethyl acetate extract was applied to the silica gel TLC plate and developed in glass chamber containing mixture of dichloromethane and methanol (9.5 : 0.5 v/v). The developed plate was visualized under short UV (254 nm), and then faced down onto the inoculated LB agar plate and incubated at 37°C for 12 hours. As a result, the inhibition zones was formed, broadly (Figure 14 (A)).

Streptomyces sp. MBL39 was culture in 100ml of YPM medium (mannitol 4g/L, yeast extract 2g/L, peptone 2g/L) and extracted with same volumn of ethyl acetate. The *in vitro* antimicrobial activities of ethyl acetate extract from *Streptomyces* sp. MBL39 were determined by MIC(minimum inhibitory concentration) test which are twofold serial microdilution method using two strains of bacteria (Gram-positive : *Staphylococcus aureus* ATCC 6538P, Gram-negative : *Proteus hauseri* NBRC 3851) at a concentration of 128 μg/ml. Extract from *Streptomyces* sp. MBL39 displayed strong inhibition activity against two strains of bacteria with MIC value of 0.8 μg/ml, equally (Figure 14 (B)).

Figure 14. **Antimicrobial test.**

(A) TLC-bioautography overlay assay of *Streptomyces* sp. Act1085.

(B) MIC test of *Streptomyces* sp. MBL39 ethyl acetate extract.

(A)

EtOAc extract	MIC value (μg/ml)	
	S. aureus	*P. hauseri*
Streptomyces sp. MBL39	0.8	0.8
Ampicillin	0.4	0.4

Staphylococcus aureus ATCC 6538P
Proteus hauseri NBRC 3851
Ampicillin was used as positive control

(B)

2. Identification of *Streptomyces* sp. Act1085 and *Streptomyces* sp. MBL39 by 16S rRNA gene sequence analysis

Total genomic DNA extracted from *Streptomyces* sp. Act1085 and *Streptomyces* sp. MBL39 was used as a template for amplification of a conserved region of 16S rRNA gene. This PCR amplification was performed by F27 and R1525 priemrs (Lane, 1991) and cloned into pGEM-T Easy Vector. After sequence analysis, coincidently, the sequence of two amplified 16S rRNA genes was the same. Also two cell lines of cultural and morphological characteristics, which produced red-purple substrate mycelium were very consistent (Figure 13 (A)). Sequence similarity searching within NCBI BLAST to identify related 16S rDNA sequences showed 100% identity with *Streptomyces californicus* NBRC 12750 (GenBank accession number AB184116.2) (Figure 15 (D)). It was also requested the microbial identification services to Charles River Laboratories Korea (Incheon, Korea) for comparison the results (Figure 15 (E)). According to a recent study using multilocus sequence analysis and DNA–DNA hybridization, it was revealed that *Streptomyces californicus* and *Streptomyces floridae* were defined as synonyms of *Streptomyces puniceus* (Rong and Huang, 2010). So, these strains were named "*Streptomyces puniceus* Act1085".

Figure 15. **Identification of *Streptomyces* sp. Act1085.**

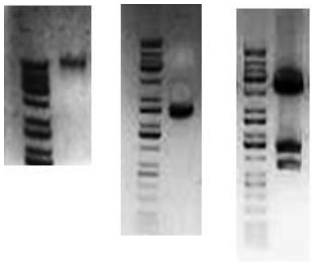
(A) Extraction of total genomic DNA of *Streptomyces* sp. Act1085.

(B) PCR with F27 and R1525 priemrs.

(C) Cloning into pGEM-T Easy vector and digestion with *EcoR* I.

(D) Sequence similarity searching wihin NCBI BLAST.

(E) Phylogenetic tree from Charles River Laboratories Korea.



(A) (B) (C)

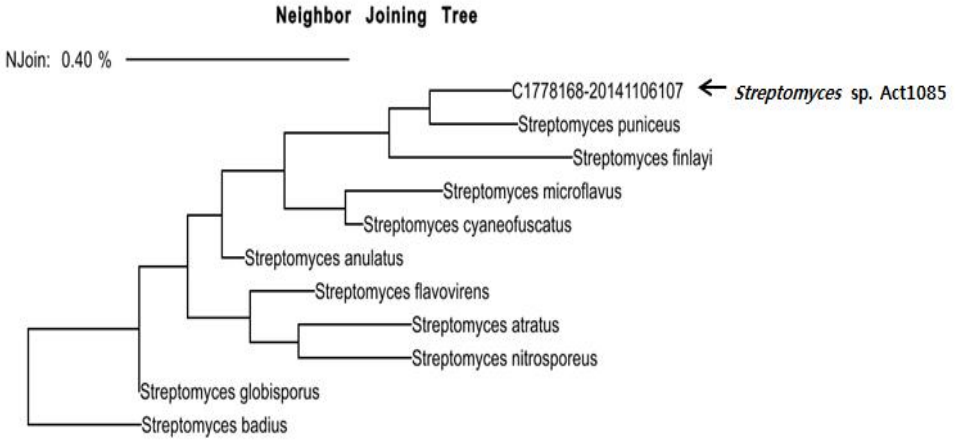
Sequences producing significant alignments:

Select: All None Selected 0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces californicus gene for 16S rRNA, partial sequence, strain: NBRC 12750	2724	2724	100%	0.0	100%	AB184116.2
Streptomyces griseus subsp. rhodochrous gene for 16S rRNA, partial sequence, strain: NBRC 13849	2724	2724	100%	0.0	100%	AB184821.1
Streptomyces californicus gene for 16S rRNA, partial sequence, strain: NBRC 3386	2724	2724	100%	0.0	100%	AB184755.1
Streptomyces sp. 82035 16S ribosomal RNA gene, partial sequence	2724	2724	100%	0.0	100%	AY996837.1
Streptomyces sp. OE53 16S ribosomal RNA gene, partial sequence	2719	2719	100%	0.0	99%	HQ392468.1
Streptomyces vinaceus gene for 16S rRNA, partial sequence, strain: NBRC 3391	2719	2719	100%	0.0	99%	AB184757.1

(D)



(E)

3. Construction of *Streptomyces puniceus* Act1085 genomic library

Streptomyces puniceus Act1085 mycelia from 30ml of YMG medium supplemented with 0.5% (w/v) glycine was used for chromosomal DNA isolation by a previously described protocol (Nikodinovic *et al*., 2003). Total genomic DNA (100μg) of *Streptomyces puniceus* Act1085 was partially digested with *Sau*3AI (total 1U) to give the optimal 30-45 kb fragments, and this optimal size fragments were ligated with *Bam*HI and *Hpa*I digested pOJ446 by using T4 DNA ligase. The ligated cosmid arms and the partially digested genomic fragments were packaged into lambda phage heads using GigaPack III XL packaging extract (Agilent Technologies) following the protocol of the manufacturer and then used to transfect *E. coli* XL1-Blue MR supercompetent cell.

Since this library contains a random population of genomic fragments that contain many overlapping genomic fragments, the number of individual clones that contain sufficient genomic DNA sequence to represent one complete genome was determined by statistical approach (Figure 16).

Figure 16. **Statistical approach of cosmid clone numbers.**

This library contains a random population of about 40kb genomic fragments that contain many overlapping genomic fragments, and the same fragment may exist. The number of individual clones that contain sufficient genomic DNA sequence to represent one complete genome was determined by statistical approach.

1 genome equivalent = total genome length / average insert size

Average *Streptomyces* genome size ≒ 8 Mb

Average insert size ≒ 40 kb

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8 Mb (=8000kb)/40 kb = 200 colonies

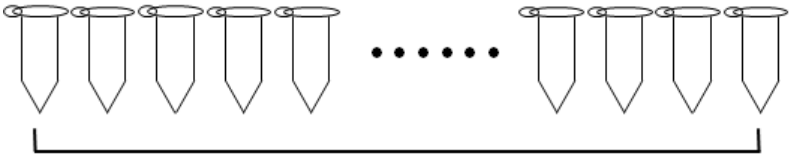
5-fold genomic redundancy because of a library containing overlapping fragments

∴ 200 colonies x 5 ≒ approximately 1,000 colonies isolation

A total of 1,020 cosmid-containing clones were separately picked and frozen at -80°C in 1.5ml eppendorf tubes as well as in pools of 30 clones consisting of 50 µl from each member of a 1.5ml eppendorf tube. Thus, the 1,020 individual cosmid-containing clones were also represented in 34 cosmid-containing pools (Figure 17).

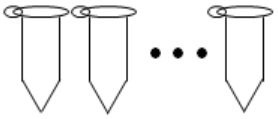
Figure 17. **1,020 individual cosmid-containing clones pooling for screening.**

One pool (1.5ml) was made up of 50 µl from 30 clones each. Thus, the 34 pools were made by collecting 1,020 individual cosmid-containing clones for PCR screening.

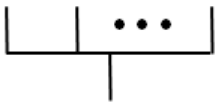


Colony inoculation (1 colony / 1 eppendorf tube)

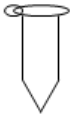
1,020 cosmid-containing clones



30 clones



50㎕ from each eppendorf tube



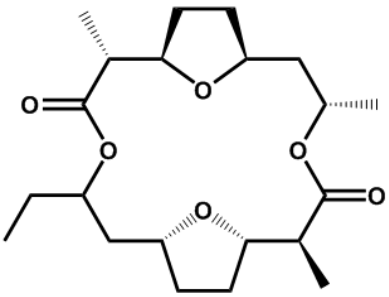
One pools
= 30 clones consisting of 50㎕ from each eppendorf tube

∴ 1,020 each clones → 34 pools for screening

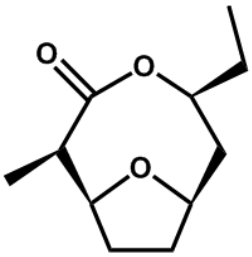
4. Screening of the cosmid library and ORFs analysis

From previous work, two nonactin derivatives, Dimeric dinactin and Cyclo-homononactic acid (Figure 18), were isolated from the culture broth of *Streptomyces puniceus* Act1085, but nonactin and other macrotetrolides were not isolated.

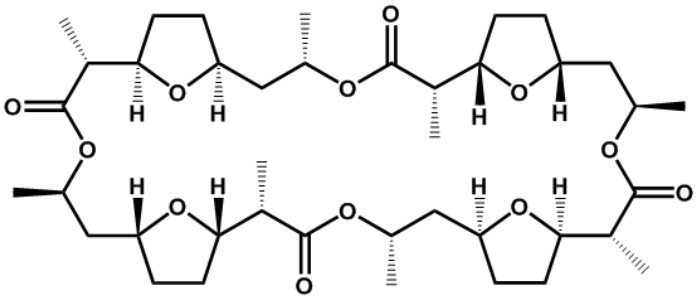
Figure 18. **Nonactin and two derivatives.**



Dimeric dinactin



Cyclo-homononactic acid



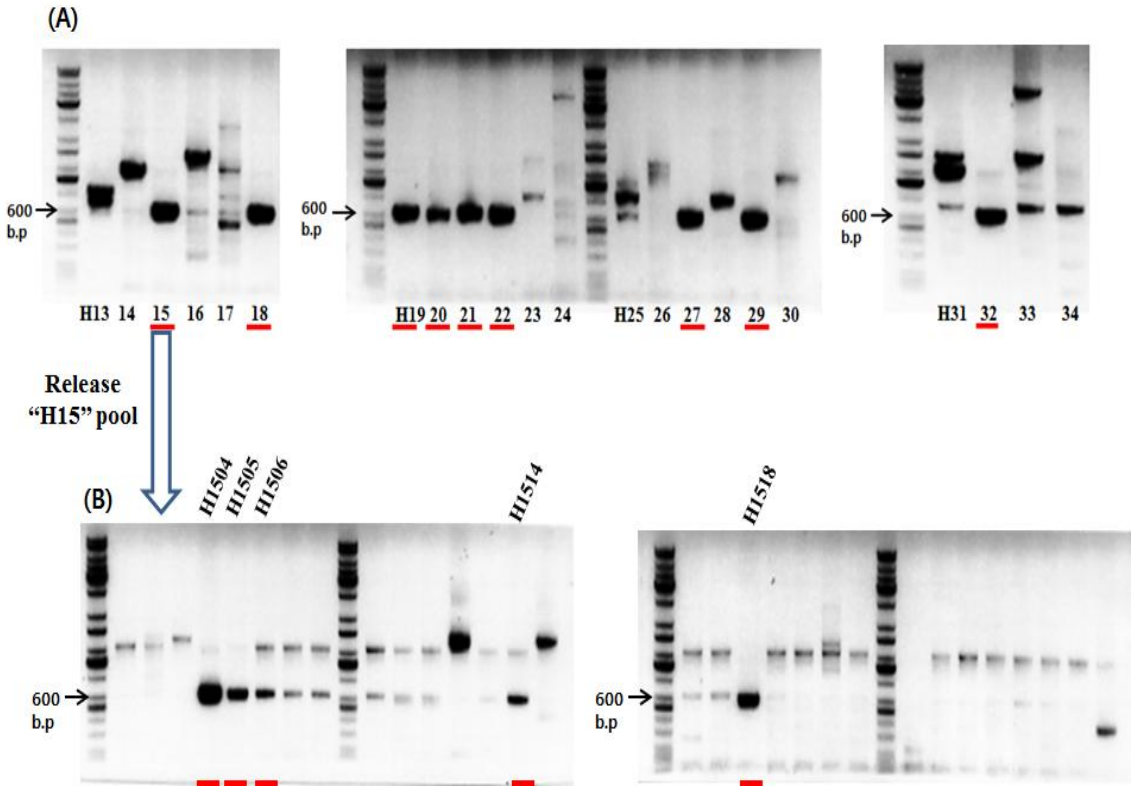
Nonactin

Therefore, it could expect that *Streptomyces puniceus* Act1085 would possess the shorter and similar nonactin biosynthetic gene cluster. To investigate if *Streptomyces puniceus* Act1085 possessed the expected genes for the synthesis of the nonactin derivatives, the cosmid library was screened by PCR amplification for those cosmids that contained *nonS*, nonactate synthase, catalyzing the formation of the furan ring from an acyclic precursor in nonactin biosynthesis (Woo *et al*., 1999). The primer sequence for nonactate synthase *nonS* referred to a deposited nonactin biosynthetic gene cluster (Walczak *et al*., 2000; GenBank accession number AF074603).

The 34 cosmid-containing pools, named these pools H1 to H34, were screened with *nonS* primers. Nine cosmid pools (H15, H18, H19, H20, H21, H22, H27, H29, H32) revealed a positive PCR fragment of the appropriate size (618 bp, Figure 19 (A)). Individual members of each *nonS*-positive cosmid pool were subsequently screened by PCR amplification to identify the specific cosmid containing putative *nonS* ortholog (Figure 19 (B)).

Figure 19. **Screening by PCR amplification by *nonS* primer.**

(A) Nine cosmid pools (H15, H18, H19, H20, H21, H22, H27, H29, H32) revealed a positive PCR fragment by *nonS* primer from 34 cosmid pools. (B) Five individual clons (H1504, H1505, H1506, H1514, H1518) revealed a positive PCR fragment from H15 pool.


(A)
600 b.p
H13 14 15 16 17 18
600 b.p
H19 20 21 22 23 24 H25 26 27 28 29 30
600 b.p
H31 32 33 34
Release "H15" pool
(B)
H1504
H1505
H1506
H1514
600 b.p
H1518
600 b.p

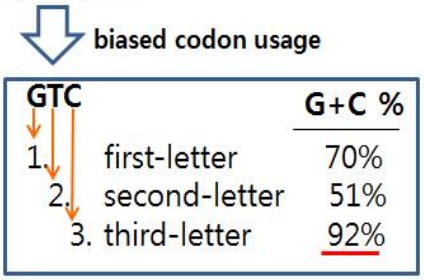
Cosmid H1504 was identified in this manner. After shotgun sequencing, the H1504 clone showed to carry an insert of 35.134 kb. Open reading frames (ORFs) were deduced from sequence data by using FramePlot 3.0 (http://www0.nih.go.jp/~jun/cgi-bin/frameplot-3.0b.pl) (Figure 20). FramePlot is a web-based tool of the ORFs analysis with genes of bacteria which have a high G+C content genome DNA such as *Streptomyces* (Ishikawa and Hotta, 1999). The graph provides for easy distinction of protein-coding regions from non-coding regions, and nucleotide and amino acid sequence (FASTA format) of the ORF you interested can be obtained via by clicking the graph.

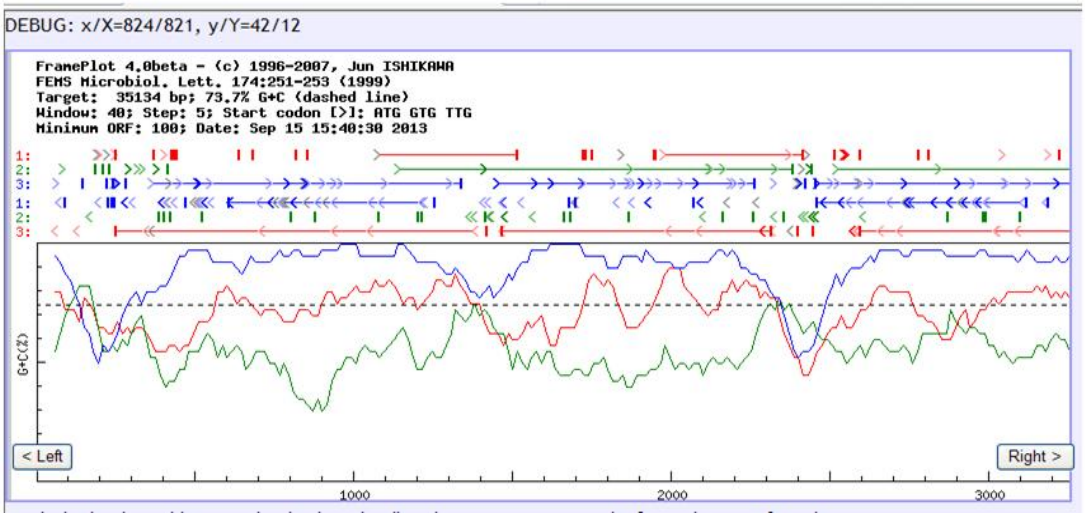
Figure 20. **Example of FramePlot analysis.**

FramePlot is a bioinformatics tool of the frame analysis for predicting protein-coding regions in bacterial DNA with a high G + C content.The third position of *Streptomyces* in each codon has a highly biased (about 90%) frequency of G and C. FramePlot utilize this bias for gene recognition.

"**FramePlot**" analysis

: FramePlot is a web-based tool for predicting protein-coding region in bacterial DNA with high G+C content, such as ***Streptomyces***





Against our expectation which *Streptomyces puniceus* Act1085 would possess the shorter gene cluster than nonactin biosynthetic gene cluster, nucleotide sequence analysis of H1054 clone revealed a very high similarity (*ca*. 89%) to nonactin biosynthetic gene cluster. The GeneBank accession numbers for the nonactin biosynthetic gene cluster are AF074603 (Walczak *et al*., 2000), AF263011 and AF263012 (Smith *et al*., 2000). Putative function of ORFs determined by comparison with other proteins using BLAST methods (http: //www.ncbi.nlm.nih.gov/BLAST/). Likewise in DNA sequences, similar results were obtained from BLAST protein database search. ORFs of H1054 clone had, from 82% to 96%, a very high amino acid sequence identity to the corresponding ORFs of the nonactin biosynthetic gene cluster from *S. griseus* subsp. *griseus* ETH A7796 (Figure 21), (Table 1).

Figure 21. **Comparison of two biosynthetic gene cluster.**

(A) Nonactin biosynthetic gene cluster from *Streptomyces griseus* DSM40695.

(Adapted from Kwon *et al*., 2001)

(B) High similar nonactin biosynthetic gene cluster from *Streptomyces puniceus* Act1085.

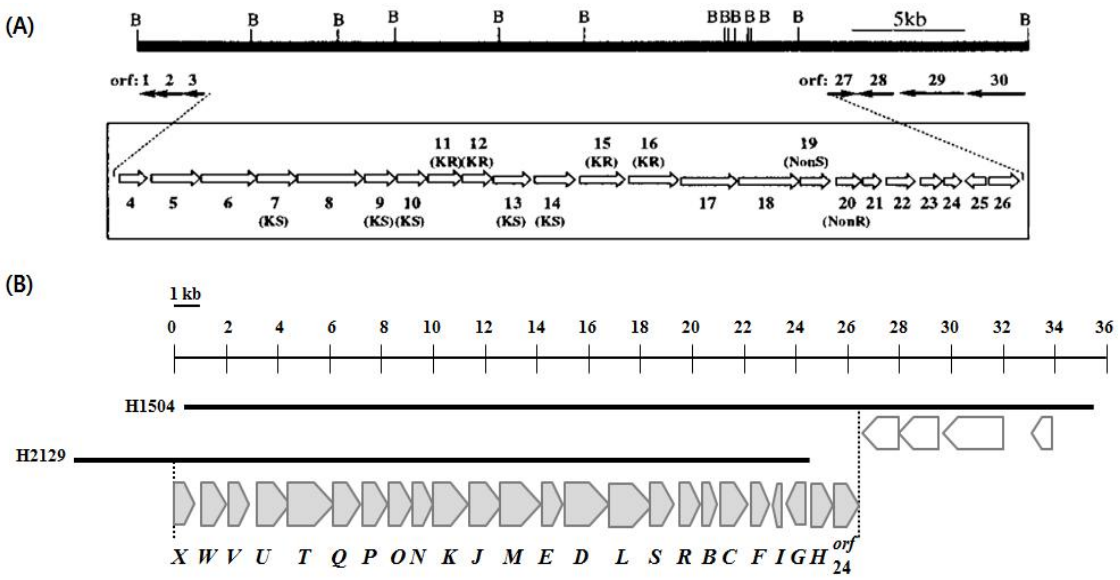

(A)
B
5kb
orf: 1 2 3
orf: 27 28 29 30
11 12
(KR)(KR)
15 16
(KR) (KR)
19
(NonS)
4 5 6 7 8 9 10 13 14 17 18 20 21 22 23 24 25 26
(KS) (KS)(KS) (KS) (KS)
(NonR)
(B)
1 kb
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
H1504
H2129
X W V U T Q P O N K J M E D L S R B C F I G H
orf 24

Table 1. Summary of cosmid H2129 and H1504 ORFs compared with nonactin genes.

ORF	No.of aa	Proposed function	Protein Homology	Amino Acid Identity(%)
orf 1	264	Enoyl-CoA hydratase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, upstream region. ORF4, NonX (AAF81231.1)	216/262 (82)
orf 2	323	ABC transporter	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, upstream region. ORF5, NonW (AAF81232.1)	275/300(92)
orf 3	268	ABC transporter	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, upstream region. ORF6, NonV (AAF81233.1)	255/268 (95)
orf 4	430	Ketoacyl synthase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, upstream region. ORF7, NonU (AAF81234.1)	386/415(93)
orf 5	594	Acetate CoA-transferase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, upstream region. ORF8, NonT (AAF81235.1)	532/595 (89)
orf 6	347	Oxoacyl-synthase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, upstream region. ORF9, NonQ (AAF81236.1)	333/347 (96)
orf 7	322	Ketoacyl synthase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, upstream region. ORF10, NonP (AAF81237.1)	294/322 (91)
orf 8	303	ketoacyl reductase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, upstream region. ORF11, NonO (AAF81238.1)	264/303 (87)
orf 9	274	Dehydrogenase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, upstream region. ORF12, NonN (AAF81239.1)	263/274 (96)
orf 10	462	Acyl-ACP synthase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonK (AAD37450.1)	391/423(92)
orf 11	426	Oxoacyl-ACP synthase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonJ (AAD37451.1)	390/426(92)
orf 12	529	ketoacyl reductase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonM (AAD37452.1)	447/533 (84)
orf 13	271	Ketoacyl-ACP reductase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonE (AAD37453.1)	243/271(90)
orf 14	569	Dipeptidyl-peptidase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonD (AAC26133.1)	517/569(91)
orf 15	555	CoA-ligase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonL (AAC26134.1)	505/553(91)
orf 16	297	Enoyl-CoA hydratase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonS (AAC26135.1)	273/296(92)
orf 17	279	Hydrolase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonR (AAD37454.1)	251/279(90)
orf 18	190	Transcriptional regulator	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonB (AAD37455.1)	149/161 (93)
orf 19	347	Aminohydrolase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonC (AAD37456.1)	283/317 (89)
orf 20	234	Thiazole biosynthesis	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonF (AAD37457.1)	215/234 (92)
orf 21	127	Hypothetical protein	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonI (AAD37458.1)	107/127 (84)
orf 22	250	Transcriptional repressor	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonG (AAD37459.1)	220/250 (88)
orf 23	276	Flavoprotein reductase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, partial sequence. NonH (AAD37460.1)	260/276 (94)
orf 24	320	Flavoprotein reductase	*Streptomyces griseus* subsp. *griseus* ETH A7796 macrotetrolide biosynthesis gene cluster, downstream region. ORF26 (AAF81241.1)	290/320 (91)

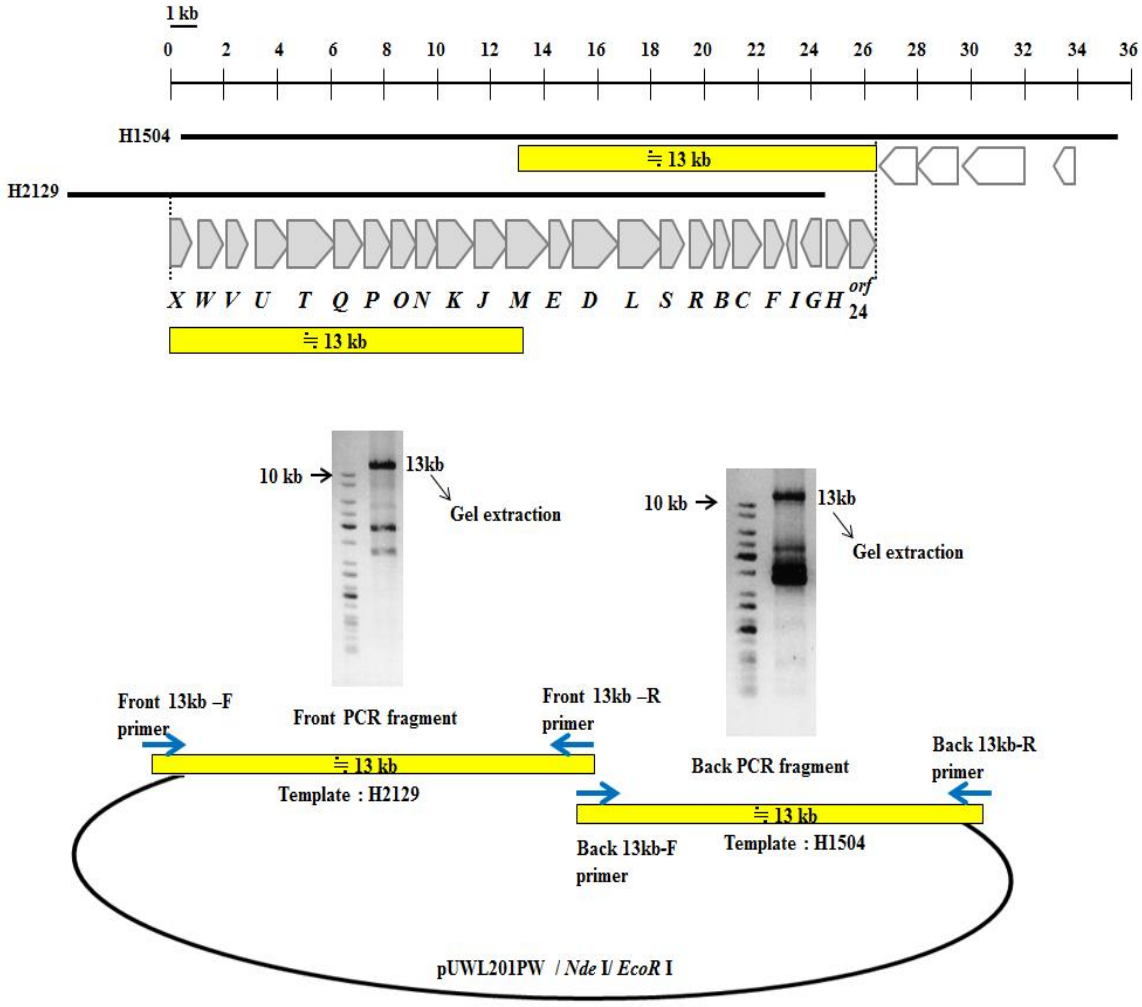
Unfortunately, the H1504 clone lacks 646 bp at its 5' end in sequence. As a results, gene cluster of H1504 clone showed a little short length as compared with nonactin biosynthetic gene cluster. The H1054 clone lacks the DNA sequence(646 bp) of the region harboring putative *nonX* ortholog (Kwon *et al*., 2002). Therefore, the another clone which contain similar genes of nonactin was found. The forward and reverse primer from *nonX* were used for library screening, and the clone revealed a positive PCR fragment by *nonX* primer were carried out End-sequencing (both ends of a insert) with cosmid pOJ446 primer. Cosmid H2129 was identified in this manner. Nucleotide sequence analysis of H2129 clone also revealed a very high similarity (*ca*. 89%) to nonactin biosynthetic gene cluster. Unfortunately, the H2129 clone lacks 775 bp at its 3' end in sequence, therefore the H2129 clone lacks the DNA sequence (775 bp) of the region harboring putative *nonH* ortholog (Figure 21 (B)).

5. Subcloning of 26 kb fragment

Although it was found that *Streptomyces puniceus* Act1085 contained a very high similar nonactin biosynthetic gene cluster, it was decided to confirm if the cluster from *Streptomyces puniceus* Act1085 produces nonactin or nonactin derivatives. However, the sequence lacks of H1504 (646 bp at its 5' end) and H2129 (775 bp at its 3' end) forced us to clone the 26 kb fragment. Because of many restriction enzyme sites in 26 kb fragment, In-Fusion cloning system (TaKaRa) was used. For the 26 kb subcloning, PCR amplification was performed to make two 13 kb PCR fragments (front and back). The front fragment PCR was performed using H2129 cosmid clone as a template, and H1504 cosmid clone was used for back fragment PCR as a template (Figure 22).

Figure 22. **Scheme of subcloning of 26 kb fragment (pXH26).**

The sequence lacks of H1504 (646 bp at the 5' end) and H2129 (775 bp at the 3' end) forced us to clone the 26 kb fragment. An *E. coli*-actinomycete expression shuttle vector, pUWL201PW, was used for this subcloning and it was digested with *Nde*I/*EcoR*I. For subcloning of the 26 kb fragment, PCR amplification was performed to make two 13 kb PCR fragments. PCR to amplify the front fragment was performed using the H2129 cosmid clone as a template with the oligonucleotides Front 13kb-F and Front 13kb-R. The back fragment PCR was performed using H1504 cosmid clone as a template with the oligonucleotides Back 13kb-F and Back 13kb-R. The linearised vector and the two amplified 13 kb PCR fragments were ligated and formed into one plasmid (pXH26) using In-Fusion Enzyme (TaKaRa) according to the manufacturer's instructions.



pXH26 construction

After finish the cloning step the plasmid harboring 26 kb fragment, named pXH26, was introduced into *S. albus* J1074 by polyethylene glycol (PEG)-mediated methods.

6. Transformation of pUWL201PW and pXH26

Two plsmids (pUWL201PW and pXH26) were introduced *Streptomyces albus* J1074 which is one of the most widely used host for the heterologous expression. The PEG-mediated transformation was carried out, since it is still the most commonly used method and a efficient technique (Figure 23).

To introduce pUWL201PW and pXH26 into *S. albus* J1074 by polyethylene glycol (PEG)-mediated methods, *Streptomyces* protoplasts were performed using standard protocols (Kieser *et al*., 2000). Spore of *S. albus* J1074 (pUWL201PW) and *S. albus* J1074 (pXH26) were cultivated in 500ml flasks containing 100ml YEME medium (yeast extract 3g/L, malt extract 3g/L, bacto-peptone 5g/L, glucose 10g/L, sucrose 340g/L) after autoclaving, supplemented with $MgCl_2 \cdot 6H_2O$ (2.5M, 2ml/L). The cultures were grown at 28°C and 220 rpm. After incubation for 7days, the cultures were extracted two times by an equal volume of chloroform and ethyl acetate.

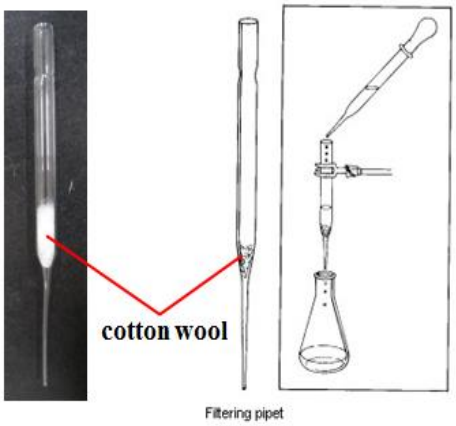
Figure 23. **Procedures of *Streptomyces* transformation.**

To introduce pUWL201PW (vector control) and pXH26 into *S. albus* J1074 by polyethylene glycol (PEG)-mediated methods, *Streptomyces* protoplasts were performed using standard protocols. First, after preparing the *S. albus* J1074 protoplasts, add up to 5 μl plasmid DNA solution to protoplasts. And then, add 200 μl 20% PEG 1000 in P buffer and spread protoplast suspension on R2YE plate. Thiostrepton is used for selection after 18 hrs.

1. Spore in YEME medium incubation 36~40h

1.1. lysozyme solution (1mg/ml P buffer)

1.2. cotton wool filtering



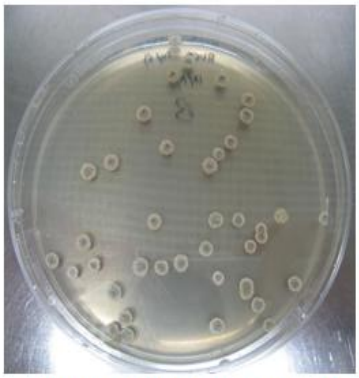
2. Protoplasts harvest

3. PEG-assisted transformation of protoplasts

3.1. add up to 5$\mu\ell$ DNA(plsmid)

3.2. add 25% PEG 1000 in P buffer

3.3. after 14-20h thiostrepton treatment by overlay



***S. albus* J1074 (pUWL201PW)**

***S. albus* J1074 (pXH26)**

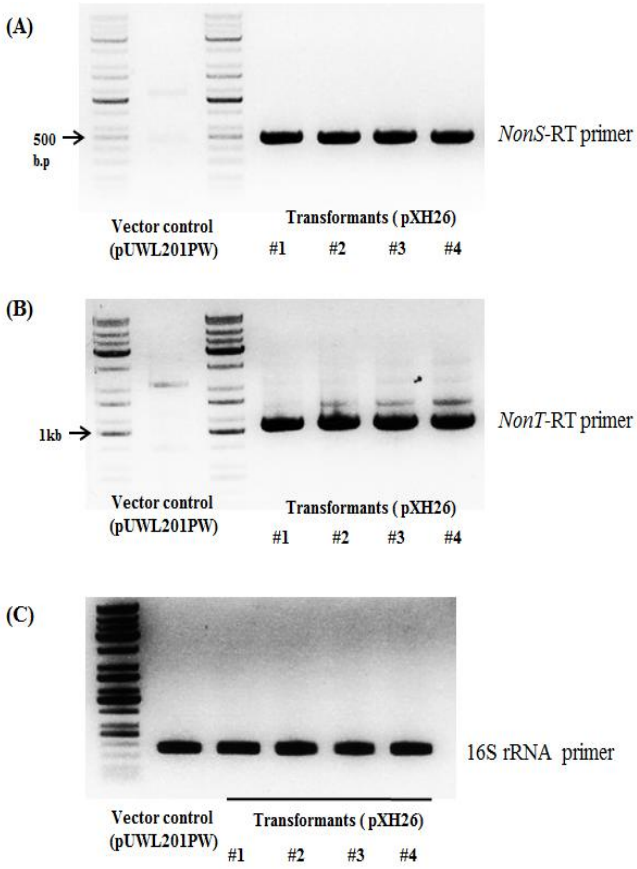
7. Reverse transcription-polymerase chain reaction of transformants

To confirm transgene expression in transformed *S. albus* J1074, the Reverse transcription-polymerase chain reaction (RT-PCR) was carried out. Total RNA isolation from *S. albus* J1074 (pUWL201PW) and *S. albus* J1074 (pXH26) transformants was conducted by TRIzol Reagent (Ambion). The 2μg of total RNA from each transformant cell lines was reverse-transcribed using Random Primer(N9), and then PCR was performed with two gene specific primers (*nonS*-RT and *nonT*-RT primer). the *S.albus* 16S rRNA gene was used as the internal control (Figure 24).

It could identify that the transgenes transformed into *S. albus* J1074 showed consecutive expression in trascript level through RT-PCR.

Figure 24. **RT-PCR of *S. albus* J1074 transformants.**

(A) RT-PCR of *S. albus* J1074 (pUWL201PW) and four *S. albus* J1074 (pXH26) transformants with *nonS*-RT primer (474 bp) (B) RT-PCR of *S. albus* J1074 (pUWL201PW) and four *S. albus* J1074 (pXH26) transformants with *nonT*-RT primer (1056 bp) (C) The *S.albus* 16S rRNA gene for the reaction control.


(A)
500 b.p
NonS-RT primer
Vector control (pUWL201PW)
Transformants (pXH26)
#1 #2 #3 #4
(B)
1kb
NonT-RT primer
Vector control (pUWL201PW)
Transformants (pXH26)
#1 #2 #3 #4
(C)
16S rRNA primer
Vector control (pUWL201PW)
Transformants (pXH26)
#1 #2 #3 #4

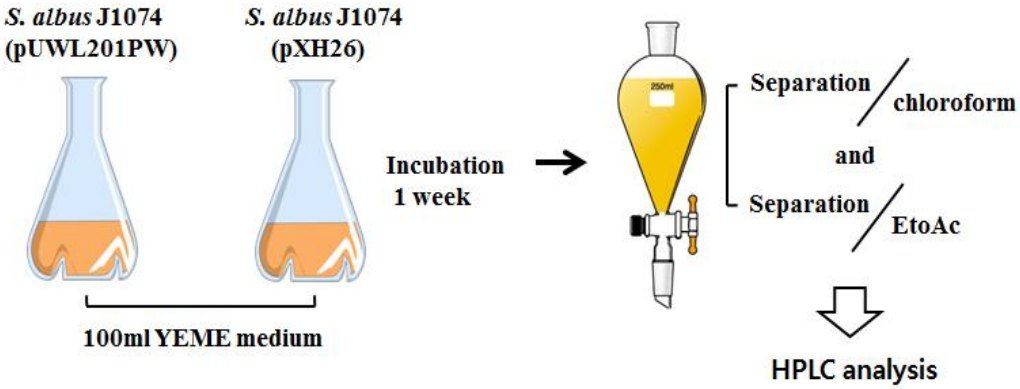
8. *S. albus* J1074 (pXH26) transformants production and analysis of compound

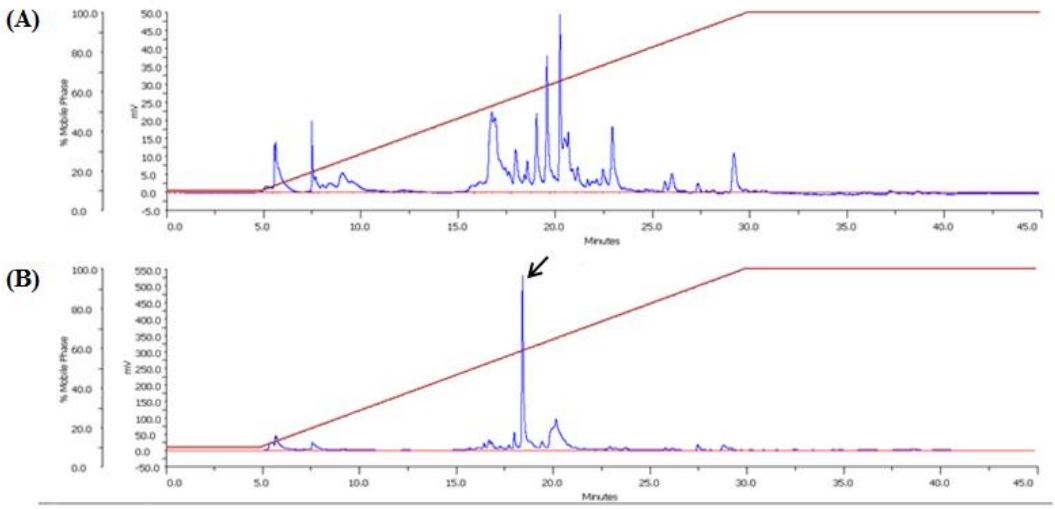
The transformants, *S. albus* J1074 (pUWL201PW) and *S. albus* J1074 (pXH26), were cultivated for 7days in 100ml YEME medium after autoclaving, supplemented with $MgCl_2 \cdot 6H_2O$ (2.5M, 2ml/L), and then the supernatant was extracted two times by an equal volume of chloroform and ethyl acetate. HPLC analysis showed that there was one strong and intense peak (arrowed peak) at *S. albus* J1074 (pXH26) chromatogram that was invisible at *S. albus* J1074 (pUWL201PW) chromatogram (Figure 25).

Figure 25. **HPLC analysis of pUWL201PW and pXH26 transformants.**

(A) *S. albus* J1074 (pUWL201PW) transformants, vector control.

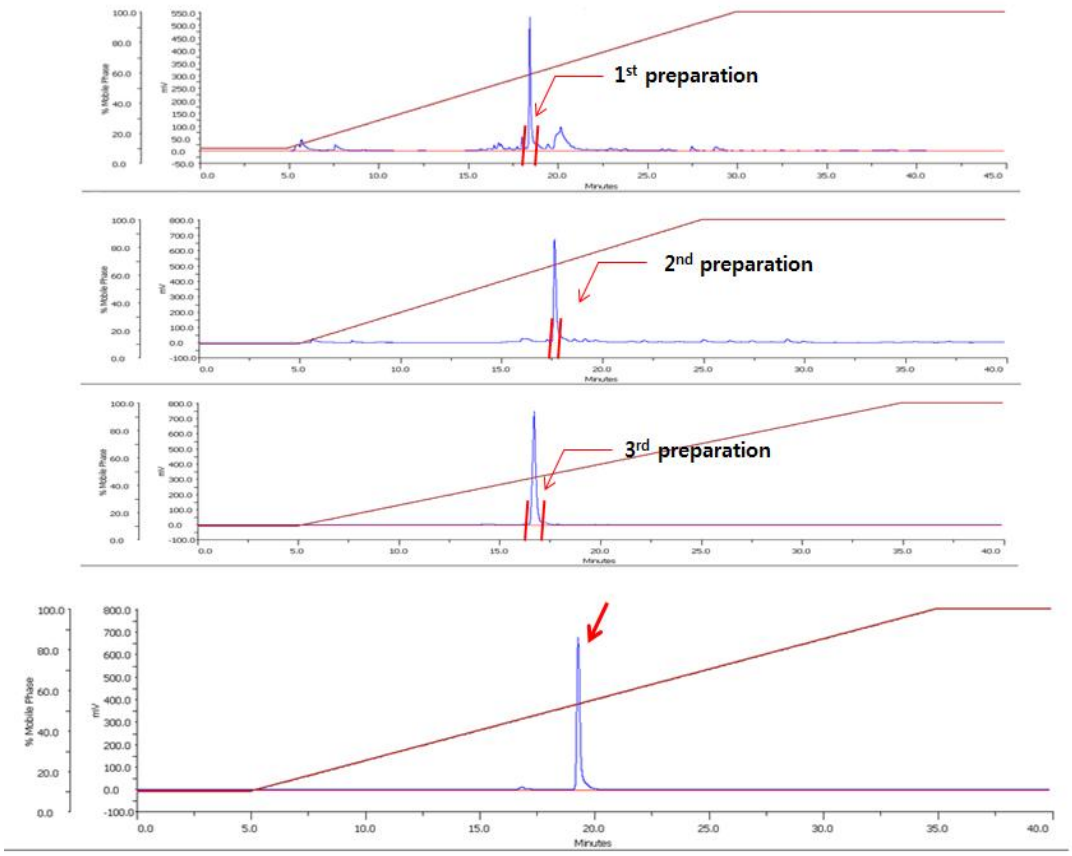
(B) *S. albus* J1074 (pXH26) transformants harboring 26 kb gene cluster .


S. albus J1074
(pUWL201PW)
S. albus J1074
(pXH26)
100ml YEME medium
Incubation
1 week
250ml
Separation
chloroform
and
Separation
EtoAc
HPLC analysis


(A)
(B)
% Mobile Phase
mV
Minutes

The one strong and intense peak (arrowed peak) was separated and purified by reversed-phase HPC with a semi-preparative column (Eclipse XDB-C18, 250 x9.4nm, 5μm particle size, Agilent) at a flow rate of 2ml/min to yield 2.8mg (Figure 26).

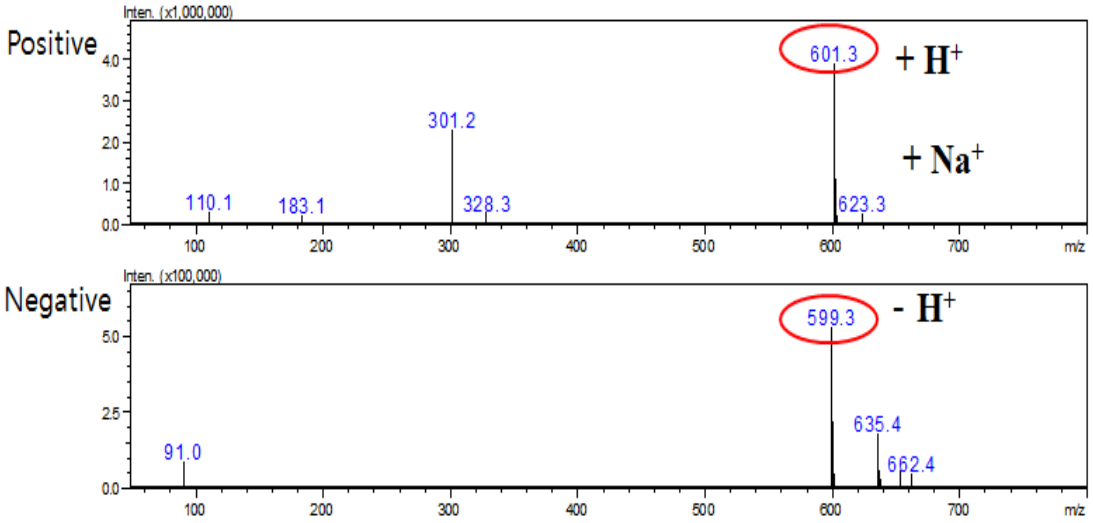
Figure 26. **Separation and purification of the peak.**


1st preparation
2nd preparation
3rd preparation
% Mobile Phase
mV
Minutes

The HPLC-MS measurement used to determine the molecular weight of an unknown substance using ESI source (Figure 27). The ESI-MS determined the molecular weight to be 601.3 $[M+H]^+$ corresponding to nocardamine (synonym: desferrioxamine E), and ^1H and ^{13}C NMR data of the purified compound also matched up with nocardamine (Supplementary materials), and were in good agreement with those in the literature (Ken Matsubara, *et al.*, 1998). ^1H NMR (500 MHz, MEOH-d_4), δ(ppm): 3.60 (t, J = 6.5, 2H), 3.16 (t, J = 6.5, 2H), 2.77 (t, J = 7.0, 2H), 2.46 (t, J = 7.0, 2H), 1.62(m, 2H), 1.51(m, 2H), 1.31 (m, 2H); ^{13}C NMR (500 MHz, MEOH-d_4), δ(ppm): 175.0, 174.5, 48.5, 40.1, 30.6, 29.8, 28.9, 27.2, 24.6.

Figure 27. **ESI-Mass spectrum of the peak.**

The HPLC-MS measurement were recorded on an LCMS-8040 (Shimadzu, Japan) instrument using an electrospray ionisation (ESI) source.


Positive
Inten. (x1,000,000)
4.0
3.0
2.0
1.0
0.0
601.3
+ H+
301.2
+ Na+
110.1
183.1
328.3
623.3
100
200
300
400
500
600
700
m/z
Negative
Inten. (x100,000)
5.0
2.5
0.0
599.3
- H+
635.4
91.0
662.4
100
200
300
400
500
600
700
m/z

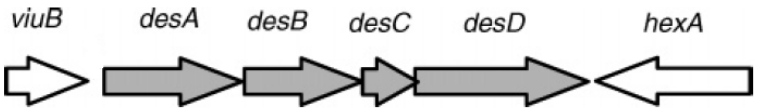
Unlike the expectation that nonactin derivatives would be produced, nocardamine was isolated from *S. albus* J1074 (pXH26) transformants harboring 26 kb gene cluster. Nocardamine is a cyclic hydroxamic acid siderophore and isolated from different *Streptomyces* strains (Keller-Schierlein and Prelog, 1961; Yang and Leng, 1982; Lee *et al*., 2005). It has been reported that nocardamine had an antibacterial activity but it was not confirmed against various strains of bacteria (Lee, *et al*., 2005), and showed anticancer activity (Brodie, *et al*., 1993; Kalinovskaya, *et al*., 2011).

The biosynthesis for nocardamine is composed of *des* operon, *desA~D*, in *S. coelicolor* M145 (Figure 28) and the biosynthetic pathway also proposed (Barona-Go´mez *et al*., 2004).

Figure 28. **Organization of the *des* operon in *S. coelicolor* M145.**

(Adapted from Barona-Go´mez *et al*., 2004)

The nocardamine biosynthesis gene cluster, which contains the *des* operon (four genes, *desA~D*), has been identified in *S. coelicolor* M145.

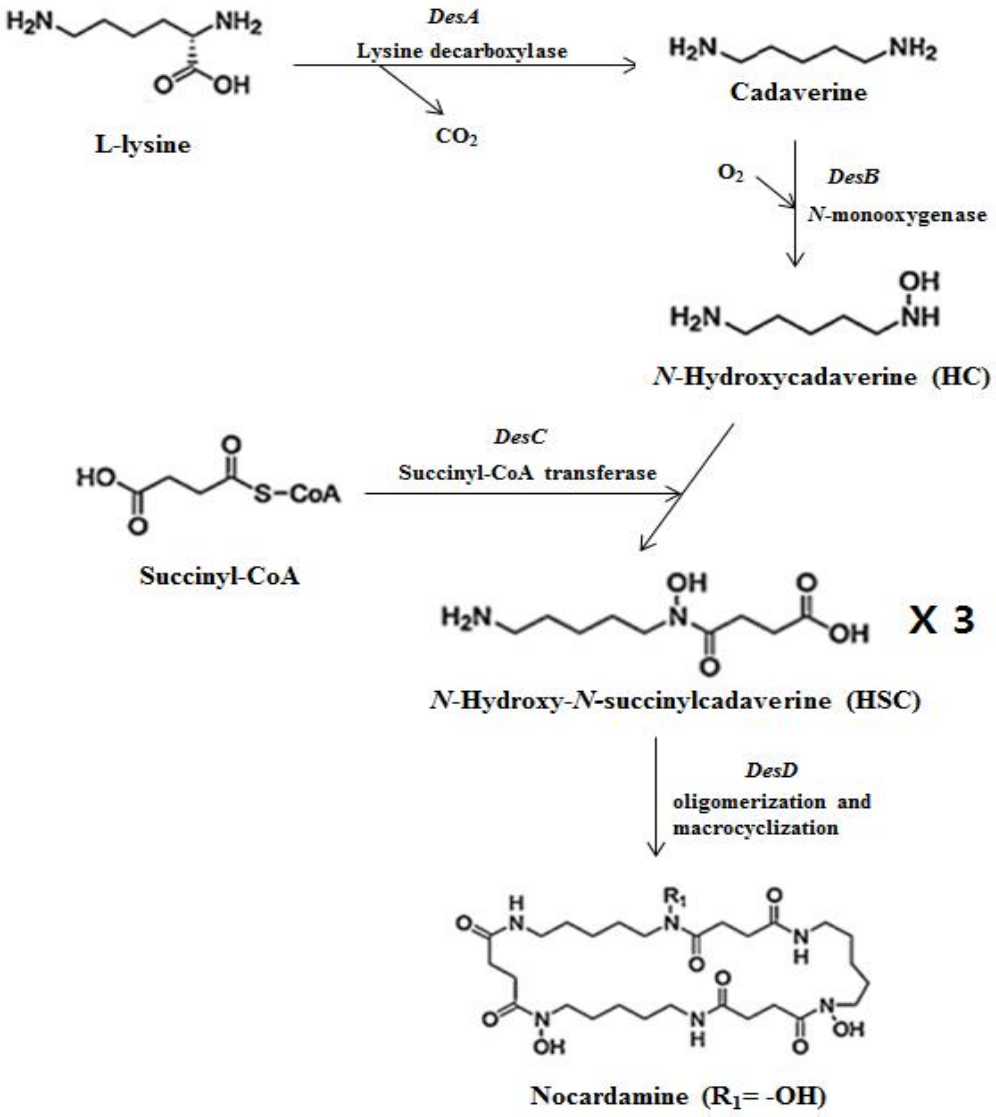

viuB
desA
desB
desC
desD
hexA

L-lysine is converted by L-lysin decarboxylase, *desA,* to cadaverine which is the first step for nocardamine biosynthesis. Cadaverine is hydroxylated by *N*-monooxygenase, *desB,* to yield *N*-Hydroxycadaverine (HC). *desC,* Succinyl-CoA transferase, is presupposed to catalyze the condensation of Succinyl-CoA and *N*-Hydroxycadaverine (HC) to *N*-Hydroxy-*N*-succinylcadaverine (HSC). Lastly, *desD* is expected to catalyze the oligomerization and macrocyclization of three *N*-Hydroxy-*N*-succinylcadaverine (HSC) to form amide bond (Barona-Go´mez *et al*., 2004) (Figure 29).

Figure 29. **Scheme of the nocardamine biosynthesis.**

(Adapted from Barona-Go´mez *et al*., 2004)

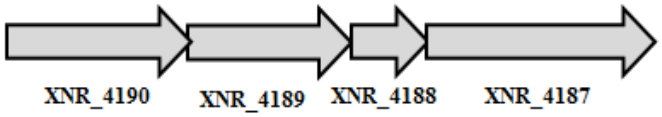
The biosynthetic pathway of nocardamine is proposed, based on the predicted function of an operon containing four genes (*desA~D*) in *S. coelicolor* M145.


DesA
Lysine decarboxylase
L-lysine
CO2
Cadaverine
O2
DesB
N-monooxygenase
N-Hydroxycadaverine (HC)
DesC
Succinyl-CoA transferase
Succinyl-CoA
X 3
N-Hydroxy-N-succinylcadaverine (HSC)
DesD
oligomerization and
macrocyclization
Nocardamine (R1= -OH)

Because of the unexpected discovery, The correlation of between nonactin and nocardamine was checked out. The *des* operon, *desA*, *desB*, *desC* and *desD*, of protein sequences were compared to nonactin biosynthetic genes. It followed that there were not any significant homology. Amino acid sequences of *des* operon genes and nonactin biosynthetic genes have very low identity (*ca.* 10~14%) in any sequence alignments. So, it was researched why a lot of nocardamine (2.8mg/100ml) were produced in the *S. albus* J1074 (pXH26) transformants harboring 26 kb gene cluster. In other *Streptomyces* strain, only make 39.0mg at 30L (Lee *et al*., 2005). The heterologous expression host, *S. albus* J1074 needed to be investigated first. Recently, complete sequence of the *S. albus* J1074 was annotated and published with a size of 6,841,649 bp, coding for 5,832 genes (Zaburannyi *et al*., 2014; GenBank accession number CP004370). The genome sequence revealed an additional 22 putative secondary metabolite gene cluster, and 2 out of 22 clusters were estimated for siderophores (Zaburannyi *et al*., 2014). After examining the two clusters, one of them had a ORFs (XNR_4190~XNR_4187**)** with protein homology to *desA*, *desB*, *desC* and *desD* (Figure 30).

Figure 30. **ORFs with homology to *des* operon in *S. albus* J1074.**

Comparison of the two siderophore biosynthetic gene clusters with the genomic database revealed that their ORFs (XNR_4190~XNR_4187) have protein homology with *desA*, *desB*, *desC* and *desD*, respectively. Deduced amino acid sequences of locus_tag XNR_4190 and *desA* had 79.38% identity. Moreover, XNR_4189, XNR_4188, and XNR_4187 showed 73.62%, 55.72%, and 69.03% identity with *desB*, *desC*, and *desD*, respectively.



XNR_4190 (485a.a) with *desA* (480 a.a), Identity 79.38% , Similarity 92.37%

XNR_4189 (436a.a) with *desB* (413 a.a), Identity 73.62% , Similarity 89.22%

XNR_4188 (201a.a) with *desC* (184 a.a), Identity 55.72% , Similarity 72.64%

XNR_4187 (606a.a) with *desD* (595 a.a), Identity 69.03% , Similarity 87.97%

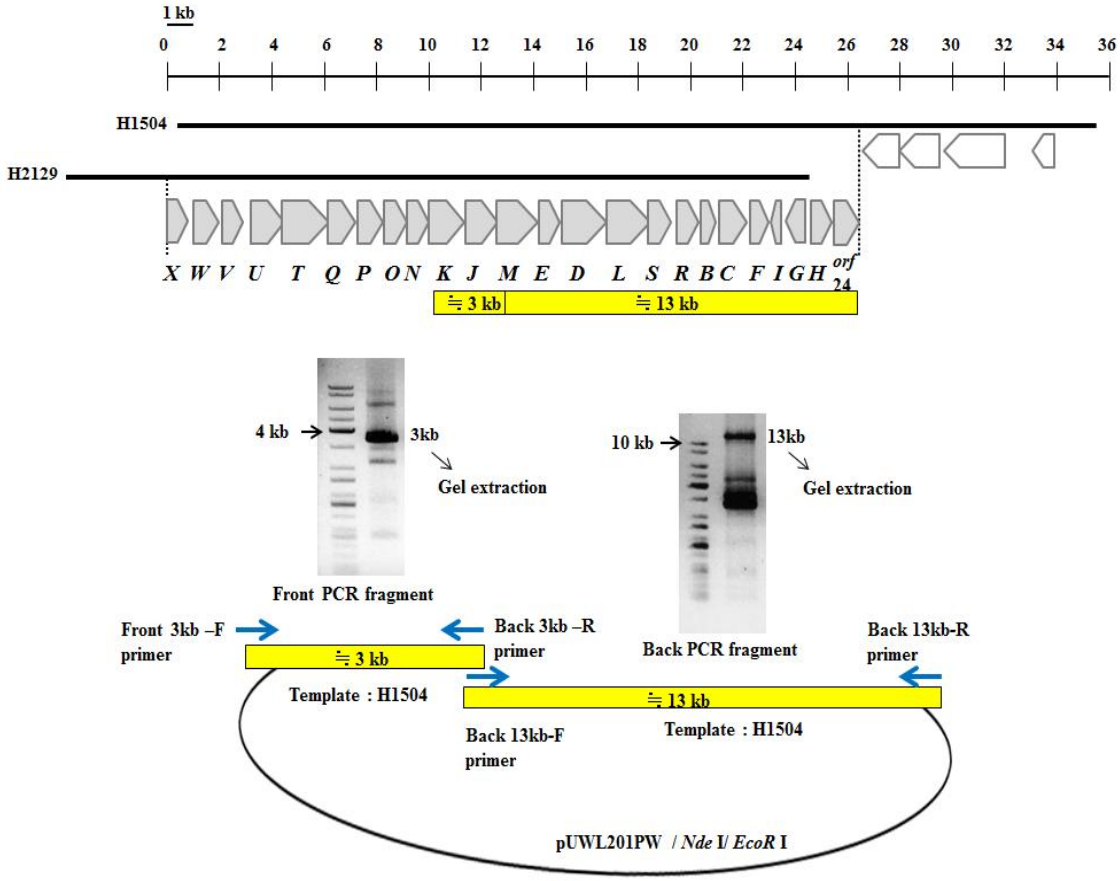
Deduced amino acid sequences of the locus_tag XNR_4190 and *desA* had 79.38% identity. Sequentially, XNR_4189, XNR_4188 and XNR_4187 showed 73.62%, 55.72%, and 69.03% identity with *desB*, *desC* and *desD,* respectively. The *Streptomyces albus* J1074 is widely used laboratory strains as a host for the heterologous expression with *Streptomyces coelicolor*, and *Streptomyces lividans,* however it possesses a large number of gene clusters for bioactive compounds *Streptomyces albus* J1074 is not known to produce any bioactive secondary metabolites (Baltz, 2010). So, the unexpected increase of nocardamine production in the *S. albus* J1074 was assumed to be due to the transformed 26 kb gene cluster. To identify which part of the 26 kb gene cluster triggers an increase in nocardamine production, the 26 kb gene cluster were divided into 16 kb (*orf 10~orf 24*) and 10 kb (*orf 1~orf 9*) (Table 1), respectively and cloned to the vector.

9. Subcloning of 16 kb and 10 kb

For the 16 kb subcloning (*orf 10~orf24*), similarly 26 kb subcloning, In-Fusion cloning system (TaKaRa) was used with two PCR fragments. The front 3 kb fragment PCR were performed using H1504 cosmid clone as a template and the back fragment PCR was reused the "13kb back fragment" from the previous 26 kb subcloning (Figure 31).

Figure 31. **Scheme of subcloning of 16 kb fragment (pXH16)**

An *E. coli*-actinomycete expression shuttle vector, pUWL201PW, was used for this subcloning and it was digested with *Nde*I/*EcoR*I. For subcloning of the 16 kb fragment, PCR amplification was performed to make 3 kb PCR fragment and 13 kb PCR fragment. PCR to amplify the 3 kb fragment was performed using the H1504 cosmid clone as a template with the oligonucleotides Front 3kb-F and Back 3kb-R. The back fragment PCR was reused 13kb back fragment from the previous 26 kb subcloning. The linearized vector, the amplified 3 kb PCR fragment and 13 kb PCR fragment were ligated and formed into one plasmid (pXH16) using In-Fusion Enzyme (TaKaRa) according to the manufacturer's instructions.

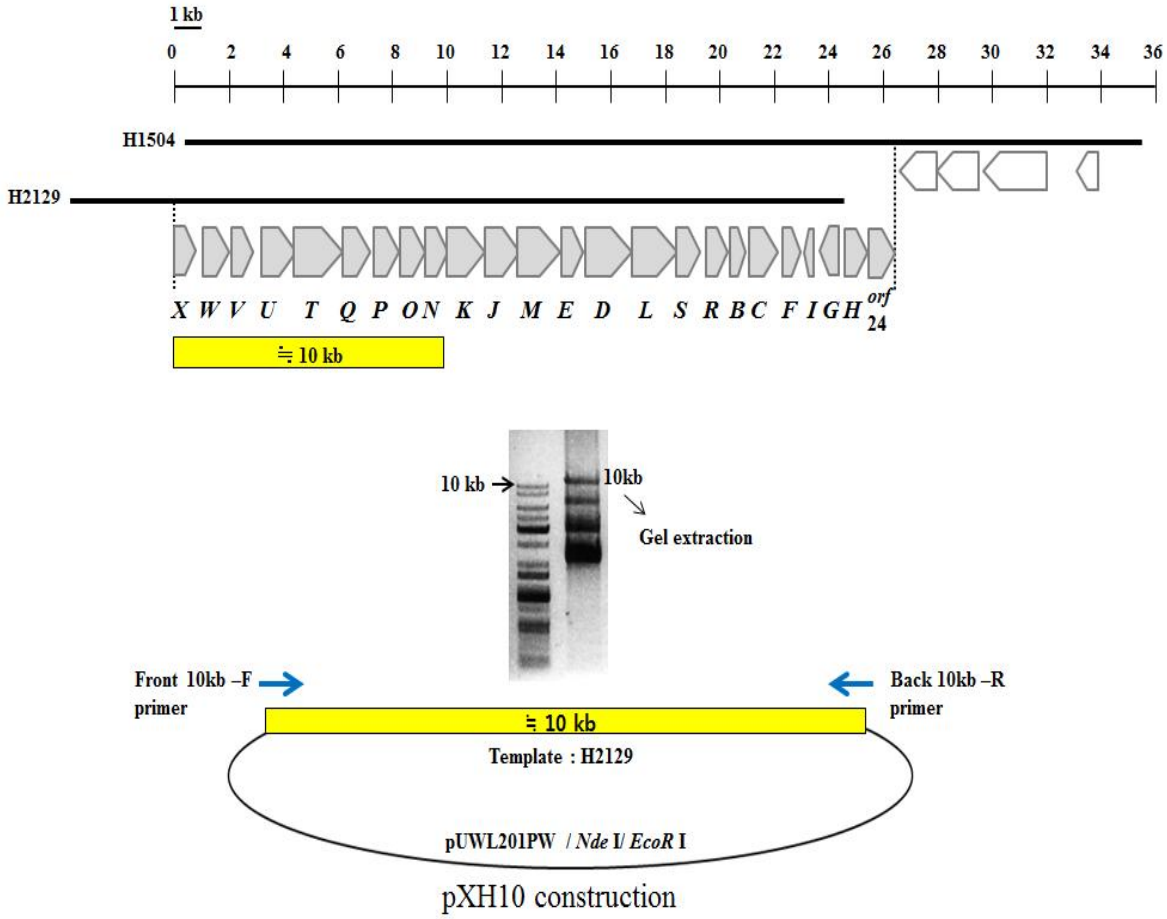


pXH16 construction

For the 10 kb subcloning (*orf 1~orf 9*), In-Fusion cloning system (TaKaRa) was also used with one PCR fragments. The 10 kb fragment PCR was performed using H2129 cosmid clone as a template (Figure 32).

Figure 32. **Scheme of subcloning of 10 kb fragment (pXH10).**

An *E. coli*-actinomycete expression shuttle vector, pUWL201PW, was used for this subcloning and it was digested with *Nde*I/*Eco*RI. For subcloning of the 10 kb fragment, PCR amplification was performed to make 10 kb PCR fragment. PCR to amplify the 10 kb fragment was performed using the H2129 cosmid clone as a template with the oligonucleotides Front 10kb-F and Back 10kb-R. The linearized vector and 10 kb PCR fragment were ligated and formed into one plasmid (pXH10) using In-Fusion Enzyme (TaKaRa) according to the manufacturer's instructions.



pXH10 construction

After finish the cloning step the plasmid harboring 16 kb fragment, named pXH16, and the plasmid harboring 10 kb fragment, named pXH10 were introduced into *S. albus* J1074, by polyethylene glycol (PEG)-mediated methods, respectively (Figure 33).

Figure 33. **Transformation of pXH16 and pXH10 to *S. albus* J1074, respectively.**

To introduce pXH16 and pXH26 into *S. albus* J1074 by polyethylene glycol (PEG)-mediated methods, *Streptomyces* protoplasts were performed using standard protocols. First, after preparing the *S. albus* J1074 protoplasts, add up to 5 μl plasmid DNA solution to protoplasts. And then, add 200 μl 20% PEG 1000 in P buffer and spread protoplast suspension on R2YE plate. Thiostrepton is used for selection after 18 hrs.

***S. albus* J1074 (pXH16)**

***S. albus* J1074 (pXH10)**

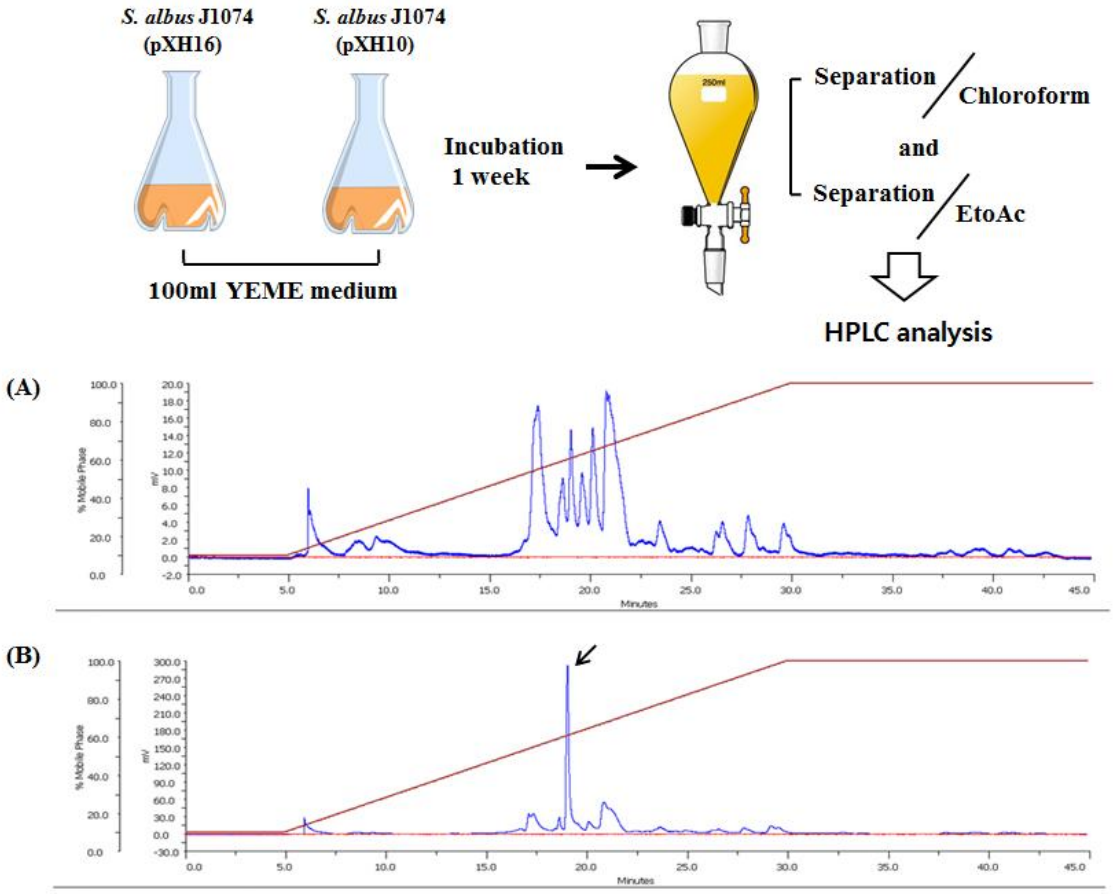
10. *S. albus* J1074 (pXH16) and *S. albus* J1074 (pXH10) transformants production

The transformants, *S. albus* J1074 (pXH16) and *S. albus* J1074 (pXH10), were cultivated in the same way as other transformants. Organic solvents extinction and HPLC analysis were also carried out in the same method as before. The strong and intense peak (arrowed peak) saw at *S. albus* J1074 (pXH26) transformant was seeen at the *S. albus* J1074 (pXH16) transformant, but it was not seen at *S. albus* J1074 (pXH10) transformant (Figure 34).

Figure 34. **HPLC analysis of pXH10 and pXH16 transformants.**

(A) *S. albus* J1074 (pXH10) transformants harboring 10 kb gene cluster.

(B) *S. albus* J1074 (pXH16) transformants harboring 16 kb gene cluster.


S. albus J1074
(pXH16)
S. albus J1074
(pXH10)
100ml YEME medium
Incubation
1 week
250ml
Separation
Chloroform
and
Separation
EtoAc
HPLC analysis
(A)
(B)
% Mobile Phase
mV
Minutes

11. Confirmation of nocardamine production regulator in nonactin biosynthetic gene cluster

Nucleotide sequence analysis of nonactin biosynthetic gene cluster are already reported, and deduced protein sequence homologies are summarized in Table 2. (The GeneBank accession numbers for the nonactin biosynthetic gene cluster are AF074603 (Walczak *et al*., 2000), AF263011 and AF263012 (Smith *et al*., 2000).

Table 2. Deduced protein sequence homologies of nonactin biosynthesis gene.

ORF	Proposed Function
nonX	enoyl-CoA dehydratase
nonW	ABC transporter ATP binding protein
nonV	ABC transporter transmembrane protein
nonU	type II beta-ketoacyl synthase
nonT	acetate CoA transferase
nonQ	oxoacy-(acyl carrier protein) synthase
nonP	type III beta-ketoacyl synthase
nonO	beta-ketoacyl reductase
nonN	shot-chain dehydrogenase
nonK	beta-ketoacyl-ACP synthase
nonJ	3-oxoacy-ACP synthase
nonM	ketoacyl reductase
nonE	3-ketoacyl-ACP reductase
nonD	Dipeptidyl-peptidase, unknown
nonL	ATP-dependent CoASH ligase
nonS	nonactate synthase
nonR	esterase, nonactin resistance
nonB	ArsR family transcriptional regulator, unknown
nonC	Aminohydrolase, unknown
nonF	Thiazole biosynthesis, unknown
nonI	hypothetical protein
nonG	tetR family transcriptional regulator, unknown
nonH	electron transfer flavoprotein beta subunit
orf24	electron transfer flavoprotein alpha subunit

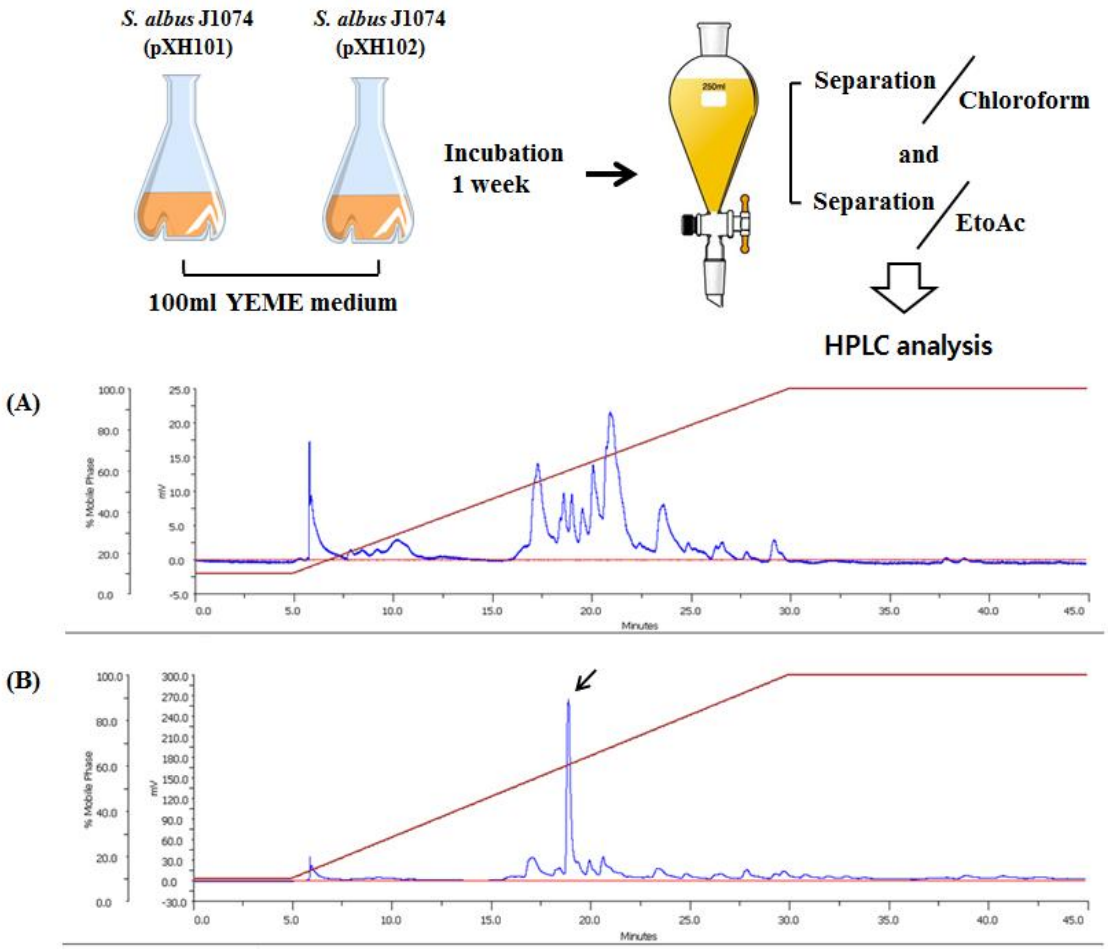
The 5 genes *nonD*, *nonB*, *nonC, nonF* and *nonG* out of 24 ORFs in nonactin biosynthetic gene cluster are not yet known for their function, exactingly. Because of the 5 genes orthologs from *S. puniceus* Act1085 are also included in the pXH16, it was anticipated that one of those 5 genes would have an effect on the increase of nocardamine production in *Streptomyces albus* J1074, and a search of related articles on 5 genes predicted that *nonB* ortholog or *nonG* ortholog from *S. puniceus* Act1085 would be related to the increase in nocardamine production. Because the two genes are highly homologous to the transcriptional regulator. Amino acid sequence identity searching of *nonB* ortholog and *nonG* ortholog wihin NCBI BLAST revealed a high identity to ArsR family transcriptional regulators and TetR family transcriptional regulators respectively (Ramos *et al*., 2005).

nonB ortholog and *nonG* ortholog from *S. puniceus* Act1085 were subcloned into same vector. The plasmid harboring *nonB* ortholog, named pXH101, and the plasmid harboring *nonG* ortholog, named pXH102 were introduced into *S. albus* J1074.The transformants were cultivated and HPLC analysis were performed in the same way as other transformants. The strong and intense peak (arrowed peak) saw at *S. albus* J1074 (pXH26) transformant (Figure 25) and the *S. albus* J1074 (pXH16) transformant (Figure 34) was seeen at the *S. albus* J1074 (pXH102) transformant (Figure 35).

Figure 35. **HPLC analysis of pXH101 and pXH102 transformants.**

(A) *S. albus* J1074 (pXH101) transformants harboring *nonB* ortholog.

(B) *S. albus* J1074 (pXH102) transformants harboring *nonG* ortholog.

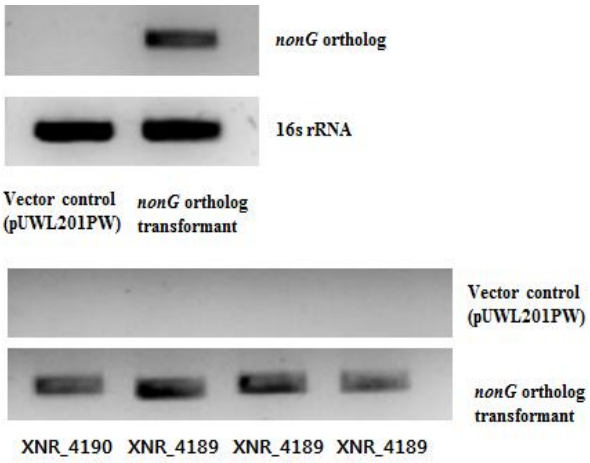

S. albus J1074
(pXH101)
S. albus J1074
(pXH102)
100ml YEME medium
Incubation
1 week
Separation
Chloroform
and
Separation
EtoAc
HPLC analysis
(A)
(B)
% Mobile Phase
mV
Minutes

12. Reverse transcription-polymerase chain reaction analysis for nocardamine biosynthetic genes expression in *S. albus* J1074

To confirm whether ORFs (XNR_4190~XNR_4187) of *S. albus* J1074, which are highly protein homology to *desA~D,* were affected by *nonG* ortholog, The transcripts of ORFs (XNR_4190~XNR_4187) was checked. Total RNA from *S. albus* J1074 (pUWL201PW) transformant and *S. albus* J1074 (pXH102) transformant was used for RT-PCR analysis. ORFs (XNR_4190~ XNR_4187) transcripts which silenced in vector control were induced in the *S. albus* J1074 (pXH102) transformant (Figure 36).

Figure 36. **Confirmation of nocardamine biosynthetic genes expression in *S. albus* J1074 (pXH102) transformant.**

Confirmation of nocardamine biosynthetic gene expressions in *S. albus* J1074 (pXH102) transformant. Total RNA from *S. albus* J1074 (pUWL201PW) transformant and *S. albus* J1074 (pXH102) transformant was used for RT-PCR analysis. The transcription levels of ORFs (XNR_4190~XNR_4187) increased in the *S. albus* J1074 cells harboring *nonG* (pXH102), but not in the cells harboring vector control (pUWL201PW).


nonG ortholog
16s rRNA
Vector control (pUWL201PW)
nonG ortholog transformant
Vector control (pUWL201PW)
nonG ortholog transformant
XNR_4190
XNR_4189
XNR_4189
XNR_4189

DISCUSSION

Actinomycetes are prolific producers of secondary metabolites, especially the marine-derived actinomycetes have a diverse, unique and novel secondary metabolites, because of massively complex and extremely different environment. The exploitation of metabolites from marine microorganisms is a rapidly growing field and providing opportunities to find new antibiotics to treat bacteria that became resistant to existing antibiotics , and it is recognized that the development of factors for activating the expression of specialized secondary metabolite biosynthetic gene clusters is important for the discovery of novel microbial natural products.

In this study, two marine-derived actinomycetes were used. *Streptomyces* sp. MBL39 was isolated from marine sediment which were collected surrounding mangrove plant's roots at the shore of Micronesia in December 2011, and *Streptomyces* sp. Act1085 received from KIOST (Korea Institute of Ocean Science & Technology). After analysis of sequence of 16S rRNA genes sequence and the morphological characteristics, both actinomycetes proved to be the same strains, and were identified as *Streptomyces puniceus*. Therefore, these strains were named "*Streptomyces puniceus* Act1085" (Figure 15).

From previous work by KIOST, two known nonactin derivatives, Dimeric dinactin and Cyclo-homononactic acid were found from the extract of the culture material of *Streptomyces puniceus* Act1085. These compounds look like a precursor of nonactin (Figure 18). Therefore, it could expect that *Streptomyces puniceus* Act1085 would possess the shorter and similar nonactin biosynthetic gene cluster. A cosmid library based on genomic DNA of the *S. puniceus* Act1085 was constructed and screened to identify a short gene cluster similar to the nonactin biosynthetic

cluster. The ORFs of the gene cluster isolated had high amino acid sequence identity, from 82% to 96%, with corresponding ORFs of the nonactin biosynthetic gene cluster from *Streptomyces griseus* subsp. *griseus* ETH A7796. (Table 1). Unlike the expectation that nonactin or nonactin derivatives would be made from *S. albus* J1074 (pXH26) transformants harboring 26 kb gene cluster, nocardamine was isolated (Figure 27). Because of the serendipity discovery, the correlation of between nonactin and nocardamine biosynthesis was checked out. Nonactin is an ionophore antibiotic produced by *S. griseus* subsp. *griseus* ETH A7796 that has shown activity against the P170-glycoprotein efflux pump associated with multiple drug resistant cancer cells (Borrel *et al*., 1994). Nocardamine is a cyclic hydroxamic acid siderophore and isolated from several *Streptomyces* strains (Keller-Schierlein and Prelog, 1961; Yang and Leng, 1982; Lee *et al*., 2005). It has been reported that nocardamine had an antibacterial activity but it was not confirmed against various strains of bacteria (Lee *et al*., 2005), and showed cytotoxicity and anticancer activity (Brodie *et al*., 1993; Kalinovskaya *et al*., 2011). The biosynthesis genes for nocardamine is composed of *des* operon, *desA~D*, in *S. coelicolor* M145 (Figure 28). The *des* operon, *desA*, *desB*, *desC* and *desD*, of protein sequences were compared to nonactin biosynthetic genes. Amino acid sequences of *des* operon genes and nonactin biosynthetic genes have very low identity (*ca.* 10~14%) in any sequence alignments. Therefore, there were not any significant amino acid sequence homology between nocardamine and nonactin biosynthetic genes. From recently published complete sequence of the *S. albus* J1074 (Zaburannyi *et al*., 2014), one siderophore biosynthetic gene cluster was found, which had a ORFs (XNR_4190~XNR_4187**)** with protein homology to *desA*, *desB*, *desC* and *desD*. Deduced amino acid sequences of the locus_tag XNR_4190 and *desA* had 79.38% identity. Sequentially, XNR_4189, XNR_4188 and XNR_4187 showed 73.62%, 55.72%, and 69.03% identity with

desB, *desC* and *desD,* respectively (Figure 30). Despite confirming the existence of nocardamine biosynthetic genes in the *S. albus* J1074, it is widely used laboratory strains as a host for the heterologous expression with *Streptomyces coelicolor* and *Streptomyces lividans*, and gene clusters for bioactive compounds in the *S. albus* J1074 is known to be silenced. Therefore, unexpected nocardamine synthesis in the *S. albus* J1074 was believed to due to the transformed 26 kb gene cluster. To identify which part of the 26 kb gene cluster triggers an increase in nocardamine production, the additional subclonings of parts of the gene cluster has been carried out. Through the additional subclonings of 16 kb and 10kb gene clusters (Figure 31 and 32), it was anticipated that *nonB* ortholog or *nonG* ortholog from *S. puniceus* Act1085 would be related to the increase in nocardamine production. Because the two genes are highly homologous to the transcriptional regulator. *nonB* ortholog and *nonG* ortholog from *S. puniceus* Act1085 were subcloned and transformed. HPLC analysis of the extract of two transformants were performed in the same way as other transformants. As a result, it was found that an unsuspected activation of nocardamine synthesis was due to the *nonG* ortholog from *S. puniceus* Act1085 for the heterologous expression host, *S. albus* J1074 (Figure 35).

To confirm whether ORFs (XNR_4190~XNR_4187) of *S. albus* J1074, which are highly protein homology to *desA~D,* were affected by *nonB* ortholog, RT-PCR was carried out. The expression of ORFs (XNR_4190~XNR_4187) increased in *S. albus* J1074 transformant harboring *nonG* (pXH102) but not in transformant harboring the control vector (pUWL201PW) (Figure 36).

The question of how dose *nonG* ortholog (TetR family trascriptional regulator) from *S. puniceus* Act1085 regulate nocardamine production in *S. albus* J1074 is important for future research. There are two possibilities. One is the indirect control method and the other is the

direct control method.

Iron-dependant repressor (DtxR and its ortholog IdeR) protein was known to regulate iron metabolism such as siderophore biosynthesis and iron uptake genes in corynebacteria, mycobacteria and streptomycetes (Ratledge and Dover, 2000; Hantke 2001). It turned out that IdeR regulates the production of nocardamine by binding to *sidABCD* operon in *S. avermitilis* K139 (Figure 37). More production of nocardamine was observed in *ideR* deletion mutant (Δ*ideR*) of *S. avermitilis* K139, and remained under higher iron concentration (Ueki *et al*., 2009).

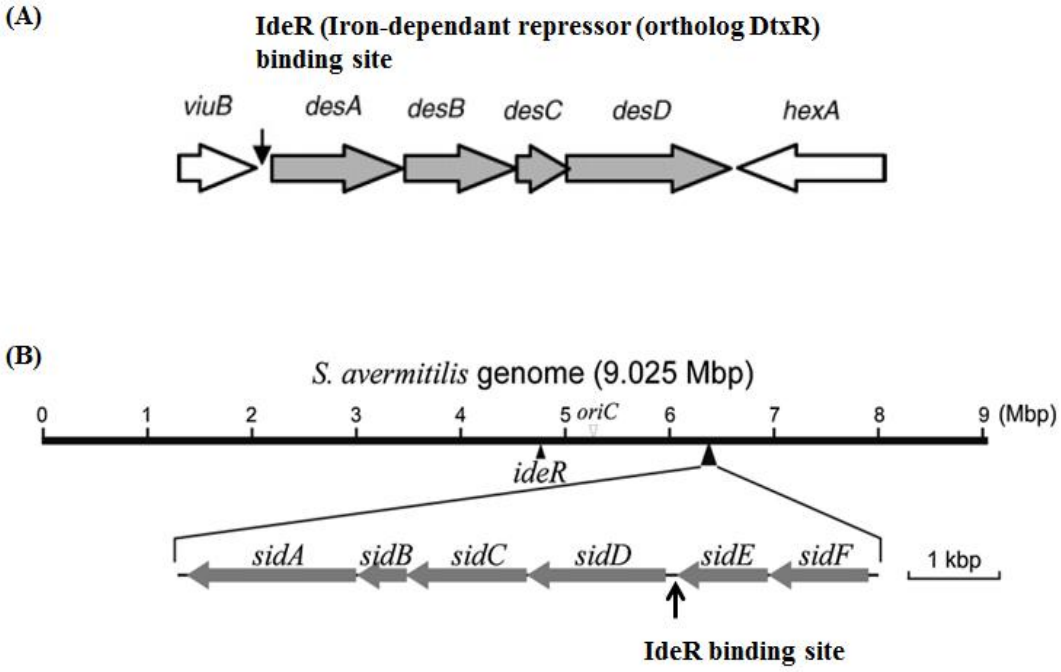
Figure 37. **Nocadamine biosynthetic gene cluster in *S. coelicolor* M145 and *S. avermitilis* K139.**

(A) The *des* operon in *S. coelicolor* M145 and IdeR binding site (arrowed site).

(B) The *sid* operon in *S. avermitilis* K139 and IdeR binding site (arrowed site).

((A) Adapted from Barona-Go´mez *et al*., 2004).

((B) Adapted from Ueki *et al*., 2009).


(A)
IdeR (Iron-dependant repressor (ortholog DtxR) binding site
viuB
desA
desB
desC
desD
hexA
(B)
S. avermitilis genome (9.025 Mbp)
0
1
2
3
4
5
oriC
6
7
8
9 (Mbp)
ideR
sidA
sidB
sidC
sidD
sidE
sidF
1 kbp
IdeR binding site

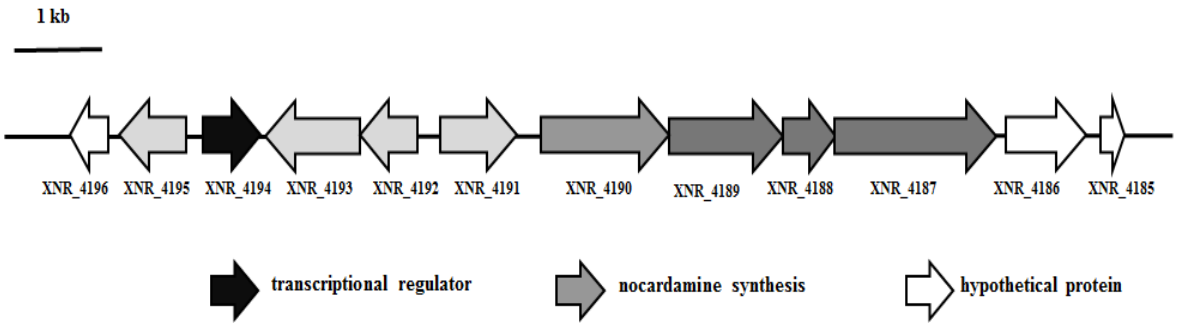
Interestingly, Iron-dependant repressor protein (IdeR or DtxR) was known not only to act in iron metabolism but also to control global gene expression including iron acquisition, storage, transcriptional regulation, acetate metabolism and fatty acid synthesis etc. Thus, *nonG* ortholog, TetR family transcriptional regulator, is expected to have the relationship with Iron-dependant repressor protein (IdeR or DtxR), not directly affeting nocardamine biosynthetic operon.

In the close distance to ORFs (XNR_4190~XNR_4187) with protein homology to *desA*, *desB*, *desC* and *desD*, several genes are found. It seems possible that these genes are involved in nocardamine biosynthesis (Figure 38).

The XNR_4191, next to the XNR_4190 gene, predicted to code for siderophore-interacting protein is in concordance with *sidE* founded in the *sid* gene cluster in *S. avermitilis* K139 (Ueki *et al*., 2009). The XNR_4194 in upstream of the ORFs (XNR_4190~XNR_4187**)** codes for a TetR-family transcriptional regulator. The presence of XNR_4194 nearby ORFs (XNR_4190~XNR_4187**)** is likely to directly influence the nocardamine gene cluster, but the function of these proteins in the nocardamine gene cluster is unclear. Therefore, it is necessary to check the relationship between transformed *nonG* ortholog and IdeR protein (indirect control), and whether XNR_4194, TetR-family transcriptional regulator, directly affects ORFs (XNR_4190~XNR_4187) (direct control) in order to determine why the silenced nocardamine biosynthesis genes are activated in the *S. albus* J1074 in the future.

Figure 38. **The close distance ORFs of the nocardamine biosynthetic gene cluster in *S. albus* J1074.**

Complete sequence of the *S. albus* J1074 was published and annotated in GenBank accession number CP004370. XNR_4191, siderophore-interacting protein; XNR_4192, lipoprotein; XNR_4193, secreted protein; XNR_4195, short-chain dehydrogenase /reductase.


1 kb
XNR_4196
XNR_4195
XNR_4194
XNR_4193
XNR_4192
XNR_4191
XNR_4190
XNR_4189
XNR_4188
XNR_4187
XNR_4186
XNR_4185
transcriptional regulator
nocardamine synthesis
hypothetical protein

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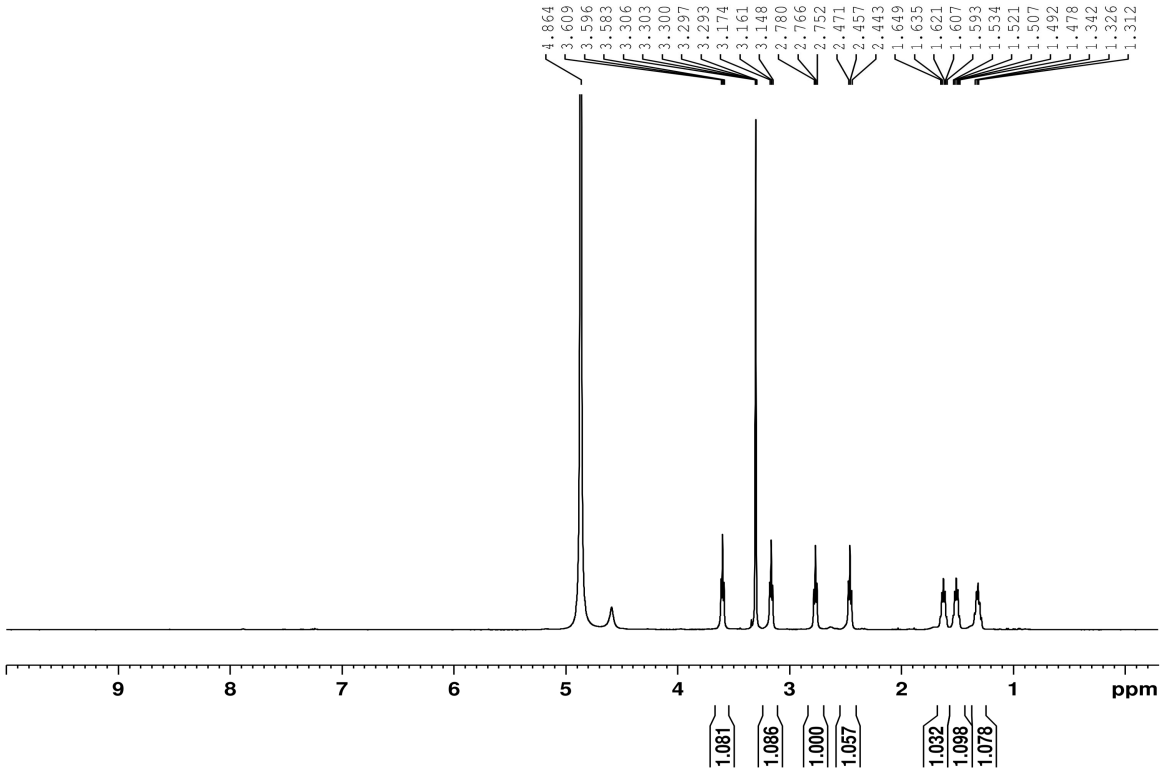
SUPPLEMENTARY MATERIALS



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PROCNO                 1

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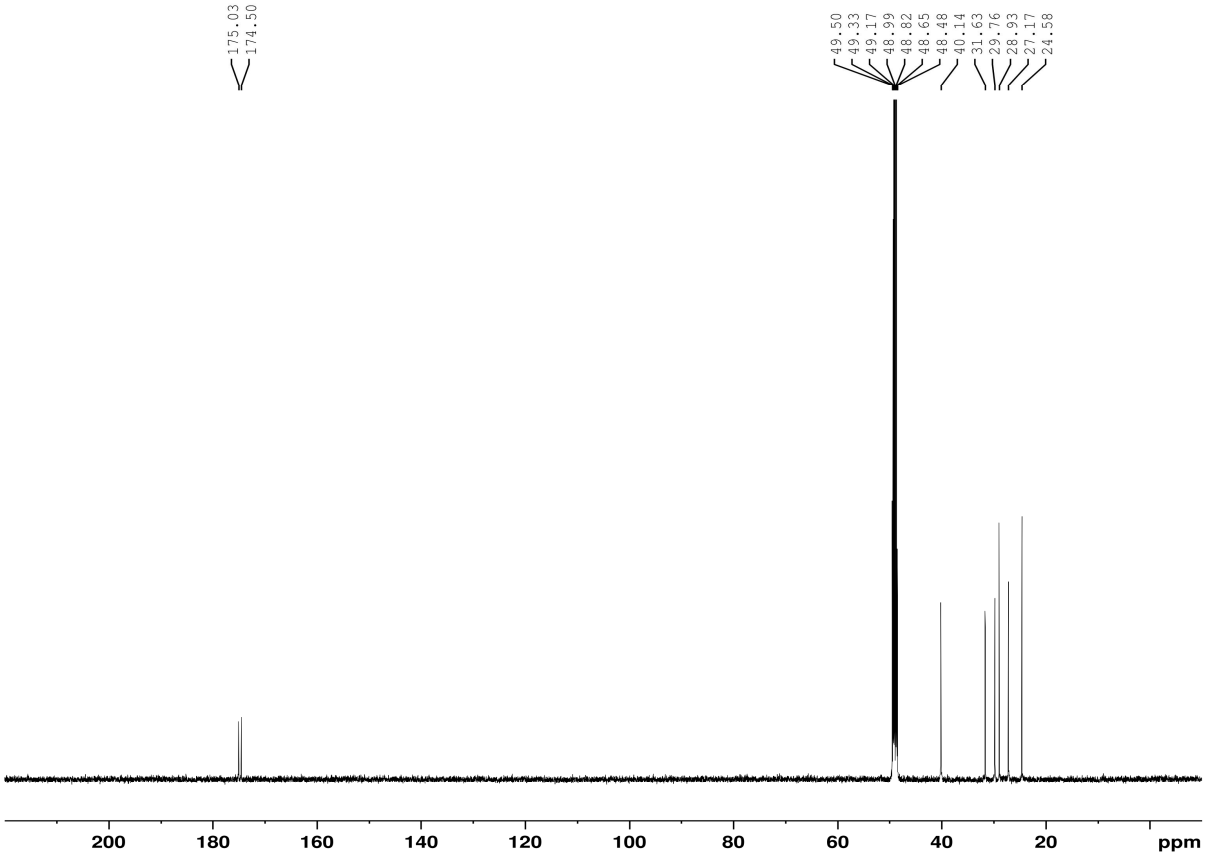
Supplementary materials. [1]H NMR spectrum of Nocardamine



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CPDPRG[2         waltz16
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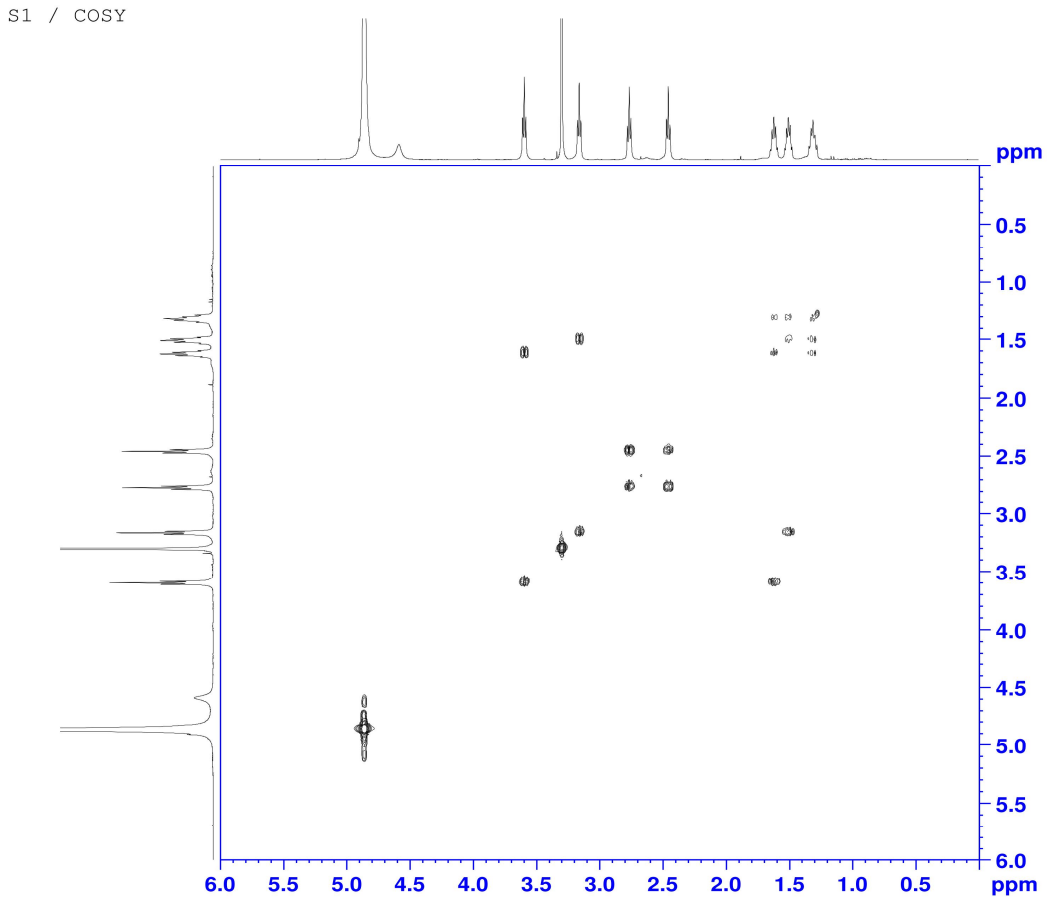
Supplementary materials. ^{13}C NMR spectrum of Nocardamine



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Current Data Parameters
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PROCNO                  1

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TD                 2048
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DS                    8
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P16              1000.00 usec

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LB          0 Hz
GB          0
PC                   1.40

F1 - Processing parameters
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GB          0
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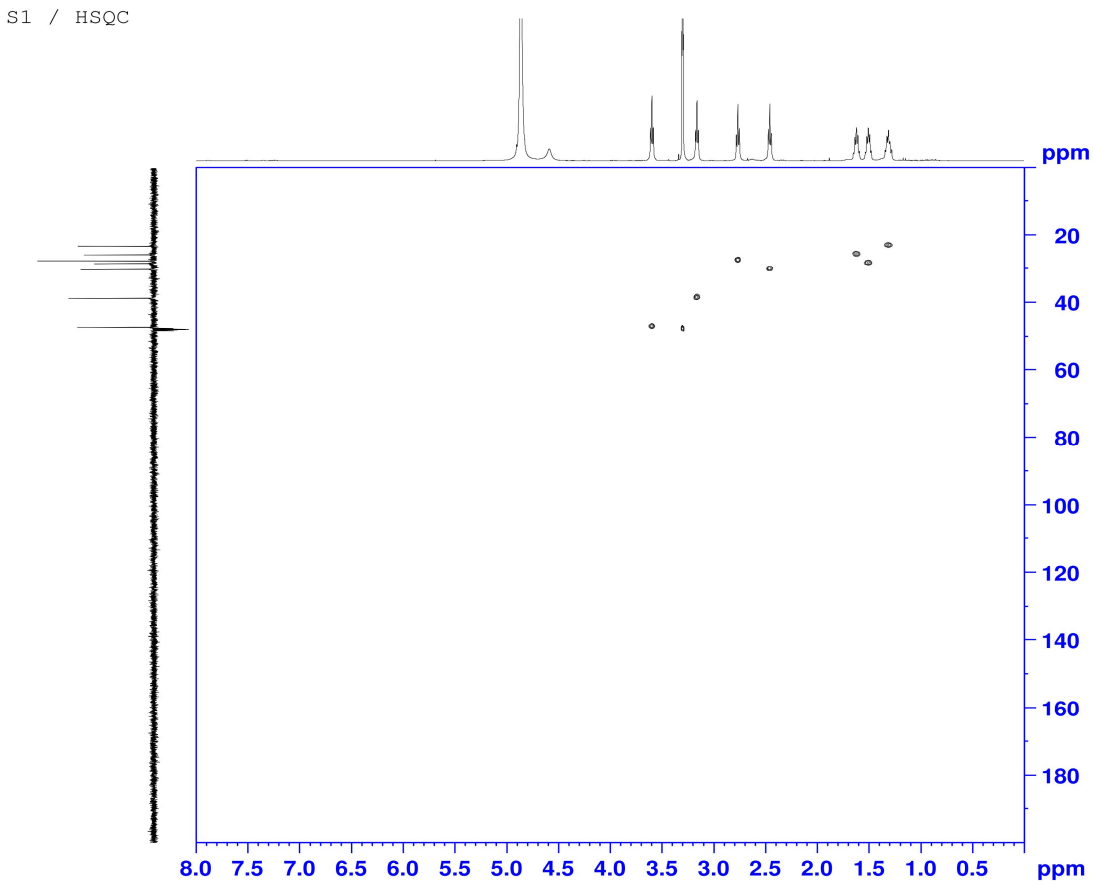
Supplementary materials. ^{1}H -^{1}H COSY spectrum of Nocardamine



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DS                     16
SWH              5580.357 Hz
FIDRES           5.449567 Hz
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TE                298.0 K
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D1            1.50000000 sec
D4            0.00172414 sec
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D21           0.00360000 sec
IN0           0.00001680 sec
TDav                    1
SFO1         500.1324506 MHz
NUC1                   1H
P1                  10.20 usec
P2                  20.40 usec
P28               1000.00 usec
PLW1         19.00000000 W
SFO2         125.7709936 MHz
NUC2                  13C
CPDPRG[2 bi_p5m4sp_4sp.2
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P14                500.00 usec
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P63               1500.00 usec
PLW0      0 W
PLW2           90.00000000 W
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SPOFFS3  0 Hz
SPW3           13.75100040 W
SPNAM[14 Crp32,1.5,20.2
SPOAL14               0.500
SPOFFS14 0 Hz
SPW14           5.86709976 W
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SPOFFS18 0 Hz
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F1 - Acquisition parameters
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SFO1          125.771 MHz
FIDRES        232.514877 Hz
SW                236.636 ppm
FnMODE     Echo-Antiecho

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SSB                     2
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GB        0
PC                   1.40

F1 - Processing parameters
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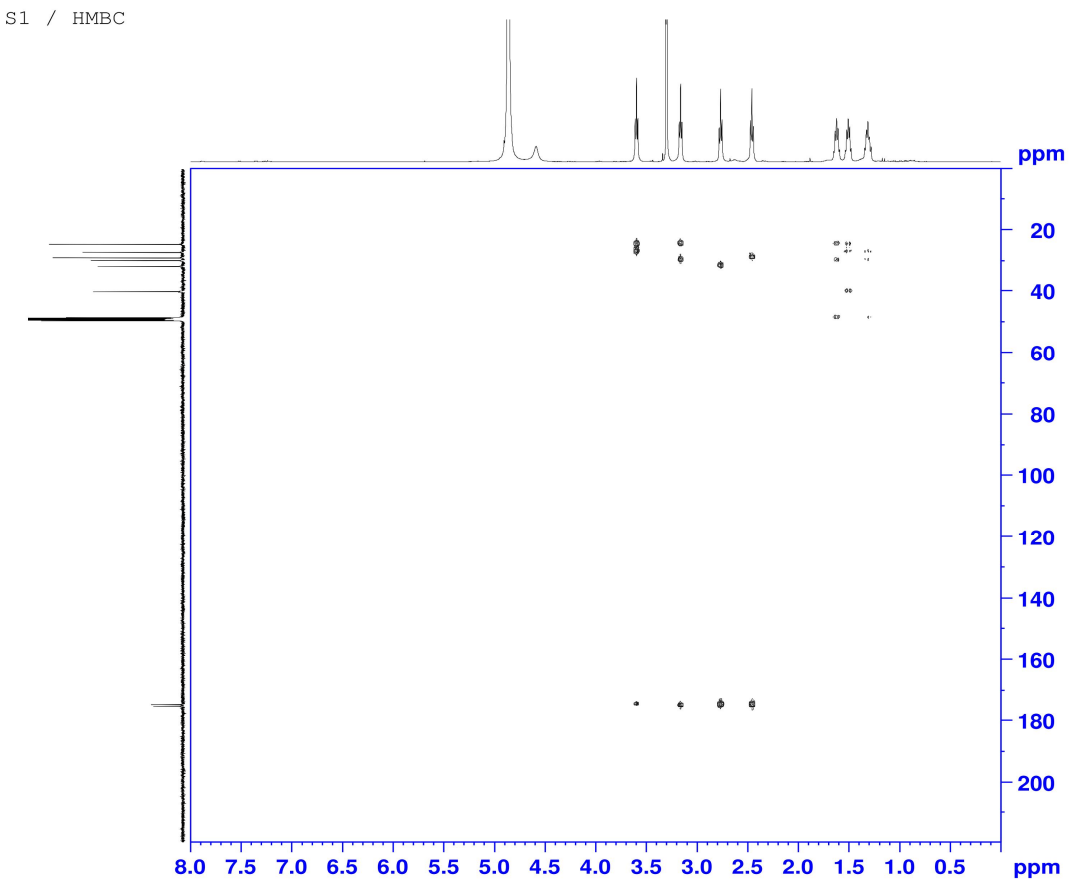
Supplementary materials. HSQC spectrum of Nocardamine



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CNST7         170.0000000
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D6              0.06250000 sec
D16             0.00020000 sec
IN0             0.00001680 sec
TDav                    1
SFO1          500.1324506 MHz
NUC1                   1H
P1                  10.20 usec
P2                  20.40 usec
PLW1          19.00000000 W
SFO2          125.7709936 MHz
NUC2                   13C
P3                  10.00 usec
P24               2000.00 usec
PLW2          90.00000000 W
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SPOFFS7   0 Hz
SPW7          13.75100040 W
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GPZ1                80.00 %
GPNAM[3]       SMSQ10.100
GPZ3                14.00 %
GPNAM[4]       SMSQ10.100
GPZ4                -8.00 %
GPNAM[5]       SMSQ10.100
GPZ5                -4.00 %
GPNAM[6]       SMSQ10.100
GPZ6                -2.00 %
P16               1000.00 usec

F1 - Acquisition parameters
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SFO1            125.771 MHz
FIDRES        232.514877 Hz
SW              236.636 ppm
FnMODE     Echo-Antiecho

F2 - Processing parameters
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SF            500.1300147 MHz
WDW                  QSINE
SSB       0
LB        0 Hz
GB        0
PC                   1.40

F1 - Processing parameters
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WDW                  QSINE
SSB                     3
LB        0 Hz
GB        0
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Supplementary materials. HMBC spectrum of Nocardamine

[Elemental Composition]
Data : FAB-0554
Sample: 210p
Note : m-NBA
Inlet : Direct
RT : 0.70 min
Elements : C 100/0, H 100/0, N 10/2, O 10/2, S 3/0
Mass Tolerance : 20ppm, 5mmu if m/z < 250, 10mmu if m/z > 500
Unsaturation (U.S.) : -10.0 - 100.0

Date : 16-Mar-2017 11:22

Ion Mode : FAB+
Scan#: (1,65)

Observed m/z	Int%	Err[ppm / mmu]	U.S.	Composition
601.3618	29.5	-8.3 / -5.0	19.0	C 40 H 47 N 3 O 2
		+12.6 / +7.6	19.5	C 39 H 45 N 4 O 2
		+0.6 / +0.4	15.5	C 33 H 45 N 8 O 3
		-1.6 / -1.0	15.0	C 35 H 47 N 5 O 4
		-3.9 / -2.3	14.5	C 37 H 49 N 2 O 5
		-13.6 / -8.2	11.0	C 29 H 47 N 9 O 5
		+7.3 / +4.4	11.5	C 28 H 45 N 10 O 5
		-15.9 / -9.5	10.5	C 31 H 49 N 6 O 6
		+5.0 / +3.0	11.0	C 30 H 47 N 7 O 6
		+2.8 / +1.7	10.5	C 32 H 49 N 4 O 7
		-9.2 / -5.5	6.5	C 26 H 49 N 8 O 8
		+11.7 / +7.1	7.0	C 25 H 47 N 9 O 8
		-11.4 / -6.9	6.0	C 28 H 51 N 5 O 9
		+9.5 / +5.7	6.5	C 27 H 49 N 6 O 9
		-13.6 / -8.2	5.5	C 30 H 53 N 2 O 10
		+7.3 / +4.4	6.0	C 29 H 51 N 3 O 10
		-2.5 / -1.5	2.5	C 21 H 49 N 10 O 10
		-13.9 / -8.4	15.0	C 37 H 51 N 3 O 2 S
		+7.0 / +4.2	15.5	C 36 H 49 N 4 O 2 S
		-5.0 / -3.0	11.5	C 30 H 49 N 8 O 3 S
		+15.9 / +9.6	12.0	C 29 H 47 N 9 O 3 S
		-7.3 / -4.4	11.0	C 32 H 51 N 5 O 4 S
		+13.7 / +8.2	11.5	C 31 H 49 N 6 O 4 S
		-9.5 / -5.7	10.5	C 34 H 53 N 2 O 5 S
		+11.4 / +6.9	11.0	C 33 H 51 N 3 O 5 S
		+1.7 / +1.0	7.5	C 25 H 49 N 10 O 5 S
		-0.6 / -0.3	7.0	C 27 H 51 N 7 O 6 S
		-2.8 / -1.7	6.5	C 29 H 53 N 4 O 7 S
		+15.9 / +9.6	6.5	C 30 H 53 N 2 O 8 S
		-14.8 / -8.9	2.5	C 23 H 53 N 8 O 8 S
		+6.1 / +3.7	3.0	C 22 H 51 N 9 O 8 S
		+3.9 / +2.3	2.5	C 24 H 53 N 6 O 9 S
		+1.7 / +1.0	2.0	C 26 H 55 N 3 O 10 S
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		-10.6 / -6.4	7.5	C 27 H 53 N 8 O 3 S 2
		+10.3 / +6.2	8.0	C 26 H 51 N 9 O 3 S 2
		-12.9 / -7.7	7.0	C 29 H 55 N 5 O 4 S 2
		+8.1 / +4.8	7.5	C 28 H 53 N 6 O 4 S 2
		-15.1 / -9.1	6.5	C 31 H 57 N 2 O 5 S 2
		+5.8 / +3.5	7.0	C 30 H 55 N 3 O 5 S 2
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		+14.4 / +8.7	7.5	C 31 H 57 N 2 O 3 S 3
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		+4.7 / +2.8	4.0	C 23 H 55 N 9 O 3 S 3

Supplementary materials. FAB-Mass analysis of Nocardamine

한글 초록

서울대학교 대학원

농생명공학부 응용생명화학전공

박완기

방선균은 자연에 널리 존재하는 미생물로써 항생제, 항곰팡이제 그리고 항암제 같은 유용한 생리활성물질을 만든다. 최근, 해양에서 유래한 방선균에 대한 연구가 많이 이루어지고 있는데 해양생태계는 육상생태계와는 생존환경이 많이 다름으로 특이한 구조의 2차대사물질을 만든다고 알려져있다. 해양에서 유래한 방선균으로부터 새로운 2차대사물질을 찾기위해, 마이크로네시아의 해양 침적물에서 *Streptomyces puniceus* MBL39 라고 명명한 방선균을 분리했고, 또한 한국해양연구원으로 부터 *Streptomyces puniceus* Act1085 라는 방선균을 분양 받았다. 두 방선균의 16S rRNA 유전자 서열과 형태적 특징을 분석한 결과, 두 방선균은 동일한 균주라는 것이 밝혀졌다. *S. puniceus* Act1085 에서는 Dimeric dinactin과 Cyclo-homononactic acid 라는 두 개의 nonactin 유도체가 발견되어, *S. puniceus* Act1085 는 아마도 nonactin biosynthetic gene cluster 와 매우 유사하면서도 크기는 좀 작은 biosynthetic gene cluster 을 가지고 있을 것이라고 예측하였다. 따라서, *S. puniceus* Act1085 가 예측한대로 그러한 biosynthetic gene cluster 을 지니고 있는 지 확인하기 위하여, *S. puniceus* Act1085 의 genomic library 을 제조하고, 적절한 probe로 screening 을 수행하였다. *S. puniceus* Act1085 에서 찾아낸 biosynthetic gene cluster 의 ORF 들을 nonactin biosynthetic gene cluster 의 ORF 들과 비교한 결과 아미노산 서열의 상동성이 82%에서 96%로 매우 높았다. *S. puniceus* Act1085 에서 찾아낸 biosynthetic gene cluster 을 이종숙주인 *Streptomyces albus* J1074 에

heterologous expression을 수행하면서, 새로운 nonactin 유도체가 만들어 질 것이라는 예상을 하였으나, nocardamine 이 생산되었다. 추가적인 heterologous expression 실험을 통해, *S. albus* J1074 에서 nocardamine 의 생산은 *S. puniceus* Act1085 에서 유래한 *nonG* ortholog 의 작용에 의한 것이라고 판단된다.

PUBLICATIONS

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