Draft genome sequence of *Actinomadura* sp. K4S16 and elucidation of the nonthmicin biosynthetic pathway

Hisayuki Komaki\(^\text{1}\), Enjuro Harunari\(^2\), Natsuko Ichikawa\(^3\), Akira Hosoyama\(^3\), Moriyuki Hamada\(^1\), Kannika Duangmal\(^4\), Arinthip Thamchaipenet\(^4\), Yasuhiro Igarashi\(^2\)

\(^1\) Biological Resource Center, National Institute of Technology and Evaluation (NBRC), Kisarazu, Chiba 292-0818, Japan.
\(^2\) Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, Imizu, Toyama 939-0398, Japan.
\(^3\) NBRC, Shibuya-ku, Tokyo 151-0066, Japan.
\(^4\) Faculty of Science, Kasetsart University, Bangkok, Thailand.

\(^\text{\#} \) Corresponding author: Hisayuki Komaki, NBRC, 2-5-8 Kazusakamatari, Kisarazu, Chiba 292-0818, Japan. E-mail: komaki-hisayuki@nite.go.jp.

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**Abstract**

*Actinomadura* sp. K4S16 (=NBRC 110471) is a producer of a novel tetronate polyether compound nonthmicin. Here, we report the draft genome sequence of this strain together with features of the organism and assembly, annotation and analysis of the genome sequence. The 9.6 Mb genome of *Actinomadura* sp. K4S16 encoded 9,004 putative ORFs, of which 7,701 were assigned with COG categories. The genome contained four type-I polyketide synthase (PKS) gene clusters, two type-II PKS gene clusters, and three nonribosomal peptide synthetase (NRPS) gene clusters. Among the type-I PKS gene (*t1pks*) clusters, a large *t1pks* cluster was annotated to be responsible for nonthmicin synthesis based on bioinformatic analyses. We also performed feeding experiments using labeled precursors and propose the biosynthetic pathway of nonthmicin.

**Key words:** *Actinomadura*, biosynthesis, nonthmicin, polyether, polyketide, tetronate

**Introduction**

Actinomycetes are well known as a promising source for diverse bioactive secondary metabolites. Especially, members of *Streptomyces* have attracted attention as the most useful screening sources for new drug leads and a large number of bioactive compounds have been identified from cultures of this genus [1,2]. Consequently, the chance of finding novel secondary metabolites from *Streptomyces* members has recently dwindled. Thus, the focus of screening has recently moved to less exploited genera of rare actinomycetes [3]. In our screening for novel bioactive compounds from rare actinomycetes, *Actinomadura* sp. K4S16 was isolated from rice field soil in Thailand and found to produce a tetronate polyether designated nonthmicin along with ecteinamycin (Fig. 1) [4]. Nonthmicin shows inhibitory activity against tumor cell invasion and protective activity for neuronal cell damage. This new polyether compound is characterized by the tetronic acid functionality modified by a chlorine atom. Halogenated tetronic acids are not known from nature except nonthmicin. In this study, we conducted whole genome shotgun sequencing of the strain to elucidate the biosynthetic pathway of nonthmicin. We herein present the draft genome sequence of *Actinomadura* sp. K4S16, together with the taxonomical identification of the strain, description of its genome properties and annotation of the gene cluster for nonthmicin biosynthesis. Biosynthetic pathway for nonthmicin was predicted by bioinformatics analysis and confirmed by precursor-incorporation experiments.

**Materials and Methods**

**Sequenced strain**

In the course of screening for novel bioactive substances from rare actinomycetes, *Actinomadura* sp. K4S16 was isolated from rice field soil collected in Thailand and found to produce a novel polyketide...
compound named nonthmicin and its known congener ecteinamycin (Fig. 1) [4]. Actinomadura sp. K4S16 was preserved as TP-A0891 at the Toyama Prefectural University, deposited into the NBRC culture collection, and publicly available from the collection as NBRC 110471.

Chemotaxonomic analyses

The isomer of diaminopimelic acid in the whole-cell hydrolysate was analyzed according to the method described by Hasegawa et al. [5]. Isoprenoid quinones and cellular fatty acids were analyzed as described previously [6].

Phylogenetic analysis based on 16S rRNA gene sequences

PCR template was prepared according to the protocol for Gram-positive bacteria of DNeasy Blood & Tissue kit (Qiagen). The gene encoding 16S rRNA was amplified by PCR using two universal primers, 9F and 1541R. After purification of the PCR product by AMPure (Beckman Coulter), the sequencing was carried out according to an established method [7]. Homology search of the sequence was conducted using EzBioCloud [8]. A phylogenetic tree was reconstructed by on the basis of the 16S rRNA gene sequence together with taxonomically close type strains showing more than 98% similarities by ClustalX2 [9].

Growth conditions and genomic DNA preparation

A monoisolate of Actinomadura sp. K4S16, isolated as single colony, was grown on polycarbonate membrane filter (Advantec) on double-diluted NBRC 227 agar medium (0.2% yeast extract, 0.5% malt extract, 0.2% glucose, 2% agar, pH 7.3) at 28°C. High quality genomic DNA for sequencing was extracted and isolated from the mycelia with an EZ1 DNA Tissue Kit and a BioRobot EZ1 (Qiagen) according to the manufacturer's protocol for extraction of nucleic acid from Gram-positive bacteria. The size, purity, and double-strand DNA concentration of the genomic DNA were measured by pulsed-field gel electrophoresis, ratio of absorbance values at 260 nm and 280 nm, and Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies), respectively, to assess the quality of genomic DNA.

Genome sequencing and assembly

Shotgun and paired-end libraries were prepared and subsequently sequenced using 454 pyrosequencing technology and MiSeq (Illumina) paired-end technology, respectively (Table 1). The 82 Mb shotgun sequences and 707 Mb paired-end sequences were assembled using Newbler v2.8 and subsequently finished using GenoFinisher [10] to yield 43 scaffolds larger than 500 bp. The draft genome sequence has been deposited in the INSDC database under the accession number BDDE01000001-BDDE01000043. The project information and its association with MIGS version 2.0 compliance are summarized in Table 1 [11].

![Chemical structures of nonthmicin and ecteinamycin.](image)

Table 1. Project information

| MIGS ID | Property | Term |
|---------|----------|------|
| MIGS 31 | Finishing quality | Improved-high-quality draft |
| MIGS 28 | Libraries used | 454 shotgun library, Illumina paired-end library |
| MIGS 29 | Sequencing platforms | 454 GS FLX+, Illumina MiSeq |
| MIGS 31.2 | Fold coverage | 8.6 x, 73 x, respectively |
| MIGS 30 | Assemblers | Newbler v2.8, GenoFinisher |
| MIGS 32 | Gene calling method | Prodigal |
| Locus tag | K4S16 |
| GenBank ID | BDDE01000000 |
| GenBank date of release | Aug, 2019 |
| GOLD ID | Not registered |
| BioProject | PRJD4748 |
| MIGS 13 | Source material identifier | NBRC 110471 |
| Project relevance | Industrial |

Genome annotation

Coding sequences were predicted with Prodigal [12] and tRNA-scanSE [13]. The gene functions were assigned using an in-house genome annotation pipeline, and domains related to polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) were searched using the SMART and PFAM domain databases. PKS and NRPS gene clusters and their domain organizations were determined as reported previously [7]. Substrates of adenylation (A) and acyltransferase (AT) domains were predicted using antiSMASH [14]. Protein-protein BLAST search against the NCBI Non-redundant protein sequences (nr) database was also used for predicting function of proteins encoded in the nonthmicin biosynthetic gene cluster.

Digital DNA-DNA hybridization

Digital DNA-DNA hybridization (DDH) between Actinomadura sp. K4S16 and A. mexicana DSM 44485T (FZNP01000001-FZNP01000053) was conducted using Formula 2 of Genome-to-Genome Distance Calculator 2.1 [15].
Feeding experiments using labeled precursors

Inoculation, cultivation, extraction, and purification were performed in the same manner as previously reported [4]. Supplementation of sodium [1-13C]acetate or [1-13C]propionate (20 mg/100 ml medium/flask, 10 flasks) was initiated at 48 h after inoculation and periodically carried out every 24 h for four times. After further incubation for 24 h, the whole culture broths were extracted with 1-butanol and several steps of purification yielded 55 mg and 100 mg of 13C-labeled nonthmicin, respectively.

Results and Discussion

Feature, classification, and genome properties

The general feature of Actinomadura sp. K4S16 is shown in Table 2. This strain grew well on ISP 2 and ISP 4 agar media, but poorly on ISP 5 and ISP 7. The color of aerial mycelia was white and that of the ISP 4 agar media, but poorly on ISP 5 and ISP 7. The strain utilized arabinose, fructose, glucose, mannitol, rhamnose, sucrose, and xylose as sole carbon source for energy and growth, but not raffinose (all at 1%).

Growth occurred at 20–45 °C (optimum 28 °C) and pH 5–8 (optimum pH 7). Strain K4S16 exhibited growth on ISP 2 agar medium. The aerial mycelium formed short chains of arthrospores. A scanning electron micrograph of this strain (Fig. 2) shows that spore chains are hooked or spiral (1 turn) and the spore surface is rugose.

The whole-cell hydrolysate of strain K4S16 contained meso-diaminopimelic acid as its diagnostic peptidoglycan diamino acid. The predominant menaquinones were identified as MK-9(H4) and MK-9(H6); in addition, MK-9(H2) and MK-9(H8) were also detected as minor components. The major cellular fatty acids (>10%) were C16:0 and C18:1 ω9c. The 16S rRNA gene sequence of the strain indicated the highest similarity (99.58 %, 1415/1421) to Actinomadura mexicana A290T (AF277195) as the closest type strain. The phylogenetic analysis confirmed that the strain belongs to the genus Actinomadura (Fig. 3).

A draft genome size of Actinomadura sp. K4S16 was 9,647,292 bp and the G+C content was 72.4 % (Table 3). Of the total 9,068 genes, 9,004 were protein-coding genes and 64 were RNA genes. The classification of genes into COGs functional categories is shown in Table 4. Digital DDH between Actinomadura sp. K4S16 and the type strain of the closest species, A. mexicana DSM 44485T suggested that the DNA-DNA relatedness was 49.0 %, which is below 70 %, the cut-off point for the assignment of bacterial strains to the same species [16]. This suggests that Actinomadura sp. K4S16 is a novel independent genomospecies.

Table 2. Classification and general features of Actinomadura sp. K4S16 [11]

| MIGS ID | Property | Term | Evidence code |
|---------|----------|------|---------------|
| Classification | Domain | Bacteria | TAS [25] |
| | Phylum | Actinobacteria | TAS [26] |
| | Class | Actinomycetales | TAS [27] |
| | Order | Actinomycetales | TAS [27-30] |
| | Suborder | Streptosporangiineae | TAS [27] |
| | Family | Thermomonosporaceae | TAS [27,30-31] |
| | Genus | Actinomadura | TAS [29,32] |
| | Species | Undetermined | This study |
| | (a new genomospecies) | strain: K4S16 | TAS [4] |
| Gram stain | Not tested | Not reported |
| Cell shape | Branched mycelia | IDA |
| Motility | Not reported | NAS |
| Sporulation | Sporulating | IDA |
| Temperature range | 20 °C to 45 °C | IDA |
| Optimum temperature | 28 °C | IDA |
| pH range; Optimum | 5 to 8; 7 | IDA |
| Carbon source | Arabinose, fructose, glucose, mannitol, rhamnose, sucrose, xylose | IDA |
| MIKS-6 | Habitat | Rice-field soil | NAS |
| MIKS-6.3 | Salinity | 0 % to 2 % NaCl | IDA |
| MIKS-22 | Oxygen requirement | Aerobic | IDA |
| MIKS-15 | Biotic relationship | Free-living | IDA |
| MIKS-14 | Pathogenicity | Not reported | NAS |
| MIKS-4 | Geographic location | Thailand | TAS [4] |
| MIKS-5 | Sample collection | March 13, 2010 | NAS |
| MIKS-4.1 | Latitude | Not reported | NAS |
| MIKS-4.2 | Longitude | Not reported | NAS |
| MIKS-4.4 | Altitude | Not reported | NAS |

a Evidence codes - IDA: Inferred from Direct Assay; TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project [33].

Figure 2. Scanning electron micrograph of Actinomadura sp. K4S16 grown on double-diluted ISP 2 agar for 7 days at 28 °C. Bar, 2 µm.
Figure 3. Phylogenetic tree based on 16S rRNA gene sequences of Actinomadura sp. K4S16 and its phylogenetically close type strains showing over 98.0% sequence similarities. Accession numbers for 16S rRNA genes are shown in parentheses. The tree uses sequences aligned by ClustalX2 [9] and constructed by the neighbor-joining method [24]. All positions containing gaps were eliminated. The building of the tree also involves a bootstrapping process repeated 1,000 times to generate a majority consensus tree, and only bootstrap values above 50% are shown at branching points. Streptospora ngium roseum DSM 43021T was used as an outgroup.

Table 3. Genome statistics of Actinomadura sp. K4S16

| Attribute                        | Value   | % of Total |
|----------------------------------|---------|------------|
| Genome size (bp)                 | 9,647,292 | 100        |
| DNA coding (bp)                  | 8,684,283 | 90.0       |
| DNA C+G (bp)                     | 6,982,736 | 72.4       |
| DNA scaffolds                     | 43       | -          |
| Total genes                       | 9,068    | 100        |
| Protein coding genes             | 9,004    | 99.3       |
| RNA genes                         | 64       | 0.7        |
| Pseudogenes                       | -        | -          |
| Genes in internal clusters       | 4,198    | 46.6       |
| Genes with function prediction    | 5,358    | 59.5       |
| Genes assigned to COGs           | 7,071    | 85.5       |
| Genes with Plam domains          | 2,655    | 29.5       |
| Genes with signal peptides       | 618      | 6.8        |
| Genes with transmembrane helices | 2,022    | 22.5       |
| CRISPR repeats                   | 1        | -          |

PKS and NRPS gene clusters in the genome

We analyzed biosynthetic gene clusters for polyketides and nonribosomal peptides in the genome. Actinomadura sp. K4S16 harbored four type-I PKS gene (t1pks) clusters, two type-II PKS gene (t2pks) clusters, and three NRPS gene (nrps) clusters, as shown in Table 5. T1pks-1 cluster encoded only a PKS composed of ACP–KS/AT/DH/KR/ACP/ACP–TE domains, which showed 87% sequence identity to phenolthiocerol synthesis type-I polyketide synthase PpsD of Mycobacterium tuberculosis 401416 (CND43678), suggesting it may synthesize phenolthiocerol-like compounds. T1pks-2 cluster encoded two PKSs whose domain organizations are KS/AT/KR and KS/AT, respectively. Since these PKSs did not show sequence similarities to PKSs whose products are identified and the domain organization is unusual, we are not able to predict the product. T1pks-3 cluster encoded a PKS composed of KS/AT/KR/DH domains. Because such domain organization is specific to iterative PKSs for enediyne syntheses, this gene cluster likely synthesizes enediyne-type polyketide compounds. T1pks-4 cluster is responsible for nontoxic synthesis as stated in the following section. T2pks-1 cluster might synthesize aromatic compounds similar to tetramycin A or mithramycin, because its KSα showed 70 to 71% sequence identities to TamM (AFY23044) and MtmP (CAA61989). T2pks-2 cluster did not show high sequence similarities (less than 55% identities) to any PKSs registered in GenBank, suggesting that the product will be unique. Nrps-1 gene cluster harbored six NRPS modules and the products were predicted to be peptides containing amino dihydroxybenzoic acid, cysteine, glycine, and methyl ornithine. Nrps-2 gene cluster encoded four modules and the products will be composed of starter molecule-Cys-Cys-methyl Cys. Nrps-3 gene cluster had seven modules and the products are likely hexapeptides including amino acid residues such as alanine and threonine. The presence of these PKS and NRPS gene clusters suggests that this strain has the potential to produce diverse polyketide- and nonribosomal peptide-compounds as the secondary metabolites.
The chemical structure of nonthmicin suggested that their carbon skeletons are assembled from five malonyl-CoA, four methylmalonyl-CoA, and three ethylmalonyl-CoA molecules by a type-I PKS pathway. We therefore searched for a t1pks cluster consisting of 12 PKS modules. Among all of the four t1pks clusters present in Actinomadura sp. K4S16 (Table 5), t1pks-4 cluster encoded six large PKSs and several enzymes related to secondary metabolite syntheses (Table 6, Fig 4a) and its assembly line contains 12 PKS modules. Substrates of AT domains in modules 1, 3 and 6 were predicted to be ethylmalonyl-CoA, whereas those in modules 5, 7, 8 and 9 were methymalonyl-CoA. According to the collinearity rule of type-I PKS pathways [17] and the chemical structure of nonthmicin, the polyketide backbone biosynthesis and polyketide backbone backbone was predicted as shown in Fig. 4b. The predicted structure is in good accordance with the nonthmicin backbone. The elongated polyketide chain is then converted to form three polyether moieties by an epoxide and epoxide hydrase/cyclase(s) in a similar manner for the nanchangmycin biosynthesis [18]. The tetrone acid part may be synthesized by ORFs K4S16_09_00680 to K4S16_09_00720 as proposed for tetrone acid-containing polyketides such as tetrorcarcin A, chlorothricin, abyssomicin, and quatromycin [19–22], because these ORFs are orthologues of TcaDs, ChlM and ChlDs, AbyAs and QunDs. Two cytochrome P450s (K4S16_09_00590 and K4S16_09_00730) and a methyltransferase (K4S16_09_00740) are probably responsible for the introduction of one hydroxy group and one methoxy group to produce ecteinamycin. Chlorination to the tetrone moiety is presumably catalyzed by a halogenase (K4S16_09_00450) to yield nonthmicin. On the basis of these bioinformatic evidences, we here propose the biosynthetic pathway of nonthmicin and ecteinamycin as shown Fig. 4b.

**Feeding experiments using labeled precursors**

To verify the predicted biosynthetic pathway for nonthmicin, feeding experiments were carried out using 13C-labeled precursors such as [1-13C]acetate and [1-13C]propionate. The signal intensities in 13C NMR spectrum of these labeled nonthmicin is shown in Table 7. Feeding of sodium [1-13C]acetate gave enrichments at twelve carbons at C4, C6, C14, C18, C20, C22, C24, C25, C26, C31, C34, and C36. [1-13C]propionate feeding enriched four methyl carbons at C28, C29, C30, and C33 (Fig. 5). These results unambiguously established that the polyether polyketide structure of nonthmicin is assembled from five malonyl-CoA, four methylmalonyl-CoA, and three ethylmalonyl-CoA. Labeling of C4 and C5 by acetate and non-labeling of C1, C2, and C3 by any precursors indicated that tetrone acid moiety is
derived from one acetate and one glycerate units [23]. These results also supported by annotated ORFs of t1pks-4 cluster (K4S16_09_00690, K4S16_09_00700, K4S16_09_00710) (Fig. 4b, Table 6).

Figure 4. Genetic map of nonthnicin biosynthetic gene cluster of Actionomadura sp. K4S16 (a) and the predicted biosynthetic pathway (b). Pink, PKS; yellow, transcriptional regulator; light blue, transporter; gray, genes related to secondary metabolite synthesis. AT, acyltransferase for malonyl-CoA; ATm, acyltransferase for methylmalonyl-CoA; ATe, acyltransferase for ethylmalonyl-CoA; kr, inactive KR.
**Table 6. ORFs of tIpkS-4 gene cluster responsible for the synthesis of nonthnicin**

| ORF | Size (aa) | Deduced function | Closest protein homolog [origin] | Id/Si (%) | Accession number |
|-----|-----------|------------------|---------------------------------|----------|-----------------|
| 00450 | 551 | halogenase | halogenase B [Actinoplanes sp. ATCC 53002] | 56/72 | AKQ04685 |
| 00460 | 188 | flavin reductase | flavin reductase-like, FMN-binding [Saccharopolyspora erythraea NRRL 2338] | 47/62 | CAM04194 |
| 00470 | 220 | two-component system response regulator | response regulator receiver protein, partial [Microbispora rosea] | 65/78 | WP_003509695 |
| 00480 | 353 | two-component system histidine kinase | hypothetical protein [Herbidiopora cretae] | 43/63 | WP_003450128 |
| 00490 | 906 | transcriptional regulator | ATPase [Microbispora sp. ATCC PTA-5024] | 41/54 | ETK35445 |
| 00500 | 576 | 3-hydroxybutyryl-CoA dehydrogenase | 3-hydroxybutyryl-CoA dehydrogenase [Streptomyces rapamycinicus NRRL 5491] | 56/65 | AGP59282 |
| 00510 | 340 | 3-oxoacyl-ACP synthase | 3-oxoacyl-ACP synthase III [Streptomyces sp. C] | 61/70 | EFL20299 |
| 00520 | 4,859 | polypetide synthase | polypetide synthase [Streptomyces albus] | 54/64 | AEZ35945 |
| 00530 | 442 | crotonyl-CoA reductase | NADP-dependent quinone reductase [Streptomyces albus PD-1] | 74/83 | EXU89989 |
| 00540 | 258 | type-II thioesterase | thiosterase [Streptomyces sp. C] | 59/69 | EFL20221 |
| 00550 | 224 | transcriptional regulator | hypothetical protein [Actinomadura madurae] | 54/70 | WP_021595170 |
| 00560 | 310 | ABC transporter ATP-binding protein | hypothetical protein [Lechevalieria aerosolanus] | 77/86 | WP_03471487 |
| 00570 | 531 | ABC transporter permease protein | hypothetical protein [Actinoplanomurpa alba] | 60/74 | WP_025598181 |
| 00580 | 388 | transcriptional regulator | LuxR-family transcriptional regulator [Actinomadura sp. EG49] | 42/52 | EWC36761 |
| 00590 | 398 | cytochrome P450 | cytochrome P450 [Streptomyces bischheggenensis BCW-1] | 52/68 | ADQ34501 |
| 00600 | 136 | epoxide hydrolase/cyclase | epoxide hydrolase [Streptomyces longisporoflavus] | 53/67 | ACR05076 |
| 00610 | 467 | epoxidase | hypothetical protein SBI_01389 [S. bischheggenensis BCW-1] | 58/70 | ADQ34510 |
| 00620 | 183 | epoxide hydrolase/cyclase | putative epoxide hydrolase/cyclase [Streptomyces albus subsp. albus] | 53/66 | CC31907 |
| 00630 | 3,941 | polypetide synthase | lasalicid modular polypetide synthase [Streptomyces sp. C] | 54/64 | EFL20211 |
| 00640 | 3,165 | polyketide synthase | polypetide synthase [Streptomyces graminifaciens] | 50/61 | BAJ16467 |
| 00650 | 263 | transcriptional regulator | putative pathway specific activator [S. longisporoflavus] | 55/67 | ACR05289 |
| 00660 | 1,563 | ABC transporter permease protein | Beta-ketoacetyl synthase [Streptomyces violaceoniger Tu 4113] | 50/62 | AEM87323 |
| 00670 | 576 | ABC transporter permease protein | Putative exporter of polyketide antibiotics-like protein | 45/60 | ACZ200124 |

| 00680 | 342 | 3-oxoacyl-ACP synthase | 3-oxoacyl-ACP synthase [Streptomyces sp. CNQ665] | 65/77 | WP_027767626 |
| 00690 | 637 | glycerol-ACP biosynthesis protein | methyoxymycolyl-ACP biosynthesis protein FbkH [Streptomyces monomaccens] | 60/69 | WP_033040949 |
| 00700 | 75 | ACP | ACP [Amoebolastis orientalis] | 72/81 | AFJ50725 |
| 00710 | 265 | 2-oxoglutarate dehydrogenase | acetyltransferase [Streptomyces olindensis] | 66/81 | KDN76174 |
| 00720 | 365 | hydroxylase or acyltransferase | 2-oxoacid dehydrogenase/acyltransferase [Micromonospora chalcea] | 54/65 | ACR83748 |
| 00730 | 398 | cytochrome P450 | cytochrome P450 [S. bischheggenensis BCW-1] | 53/71 | ADQ34501 |
| 00740 | 288 | methyltransferase | SAM-dependent methyltransferase [Streptomyces sp. NRRL F-2890] | 46/59 | WP_030734046 |
| 00750 | 5,524 | acetyl transferase | [Streptomyces violaceoniger Tu 4113] | 50/61 | AEM94952 |
| 00770 | 2,121 | polypetide synthase | PropA2 [Streptomyces piniogenus] | 51/62 | AEZ54375 |

* locus tag number after K4S16_09; \(^{a}\) identity/similarity; \(^{b}\) encoded in the complementary strand.
Precursor-directed biosynthesis of bromo-analogue of nonthmicin

A putative halogenase gene (K4S16_09_00450), showing 56% identity and 72% similarity of amino acid sequence to HalB from Actinoplanes sp. ATCC 33002, present in the nonthmicin biosynthetic gene cluster was expected to be responsible for the halogenation (Table 6). If this gene product is also active for bromine, it can be used for the precursor-directed biosynthesis of a brominated analogue. In fact, supplementation of sodium bromide into the culture resulted in the production of a new nonthmicin congener (Fig. 6a) in which the chlorine atom was replaced by the bromine atom. The structure of the bromo analogue was confirmed analysing data by MS (Fig. 6b) and NMR (data not shown).

Table 7. Incorporation of 13C-labeled precursors into nonthmicin

| Position | δC | Relative enrichments (13C-labeled) | [13C]acetate | [13C]propionate |
|----------|----|-----------------------------------|--------------|----------------|
| 1        | -86.9 | 0.78                        | 0.80         |
| 2        | -84.7 | 0.79                        | 0.64         |
| 3        | -82.1 | 0.78                        | 0.89         |
| 4        | -79.5 | 2.56                        | 0.98         |
| 5        | -76.9 | 0.82                        | 0.62         |
| 6        | -74.5 | 2.28                        | 0.84         |
| 7        | -72.1 | 0.94                        | 1.24         |
| 8        | -71.7 | 1.33                        | 4.25         |
| 9        | -70.2 | 0.86                        | 0.87         |
| 10       | -68.8 | 1.09                        | 4.85         |
| 11       | -67.4 | 1.10                        | 0.74         |
| 12       | -66.0 | 1.60                        | 4.70         |
| 13       | -64.6 | 1.06                        | 0.95         |
| 14       | -63.2 | 2.25                        | 0.89         |
| 15       | -61.8 | 0.87                        | 0.80         |
| 16       | -60.4 | 1.28                        | 4.49         |
| 17       | -59.0 | 0.80                        | 0.64         |
| 18       | -57.6 | 2.41                        | 0.88         |
| 19       | -56.2 | 1.13                        | 0.95         |
| 20       | -54.8 | 2.14                        | 0.95         |
| 21       | -53.4 | 0.71                        | 0.84         |
| 22       | -52.0 | 2.64                        | 0.90         |
| 23       | -50.6 | 0.96                        | 0.98         |
| 24       | -49.2 | 2.19                        | 1.15         |
| 25       | -47.8 | 2.49                        | 1.15         |
| 26       | -46.4 | 2.47                        | 0.89         |
| 27       | -45.0 | 1.00                        | 0.90         |
| 28       | -43.6 | 1.01                        | 0.86         |
| 29       | -42.2 | 0.97                        | 0.91         |
| 30       | -40.8 | 0.94                        | 0.75         |
| 31       | -39.4 | 3.11                        | 0.87         |
| 32       | -38.0 | 0.93                        | 0.72         |
| 33       | -36.6 | 0.86                        | 1.00         |
| 34       | -35.2 | 2.19                        | 0.96         |
| 35       | -33.8 | 0.91                        | 1.05         |
| 36       | -32.4 | 3.25                        | 0.96         |
| 37       | -31.0 | 0.82                        | 0.87         |
| 38       | -29.6 | 0.82                        | 0.93         |

*13C signal intensity of each peak in the labeled 1 divided by that of the corresponding signal in the unlabeled 1, respectively, normalized to give an enrichment ratio of 1 for the unenriched peak of C27 and C33. The numbers in bold type indicate 13C-enriched atoms from 13C-labeled precursors.

Conclusion

We successfully found the type-I PKS gene cluster for nonthmicin biosynthetic and proposed a plausible biosynthetic pathway by the genome analysis of Actinomadura sp. K4S16, a producer of nonthmicin. Incorporation experiments of 13C-labeled precursors also suggested that nonthmicin is biosynthesized by PKS pathway. These findings will provide significant information not only for the biosynthetic mechanism but also for the genetic engineering to synthesize more potential bioactive molecules based on the nonthmicin structure.

Abbreviations

A: adenylation; ABC: ATP-binding cassette; ACP: acyl carrier protein; Ala: alanine; AT: acyltransferase; ATP: adenosine triphosphate; BLAST: Basic Local Alignment Search Tool; C: condensation; CLF: chain length factor; CoA: coenzyme A; COG: Clusters of Orthologous Groups; Cys: cysteine; DH: dehydratase; DHB: dihydroxybenzoic acid; E: epimerase; ER: enoylreductase; Gly: glycine; ISP: International Streptomyces project; KS: ketosynthase; KR: ketoreductase; kr: inactive KR; MIGS: minimum information about a genome sequence; MT: methyltransferase; NBRC: Biological Resource Center, National Institute of Technology and Evaluation; NMR: nuclear magnetic resonance; NRPS: nonribosomal peptide synthetase; nrps: NRPS gene; PKS: polyketide synthase; t1pks: type-I PKS gene; t2pks: type-II PKS gene; T: thiolation; TE: thioesterase; Thr: threonine.

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Competing Interests

The authors have declared that no competing interest exists.

References

1. Berdy J. Bioactive microbial metabolites. J Antibiot. 2005; 58: 1-26.
2. Watve MG, Tickoo K, Jog MM and Bhole BD. How many antibiotics are produced by the genus Streptomyces? Arch Microbiol. 2001; 176: 386-390.
3. Lazzarini A, Cavaletti L, Toppo G and Marinelli F. Rare genera of actinomycetes as potential producers of new antibiotics. Antonie van Leeuwenhoek 2000; 78: 399-405.
4. Igarashi Y, Matsuoka N, In Y, Kataura T, Tashiro E, Saiki I, Sudo Y, Duangmal K and Thamchaipenet A. Nonthmicin, a polyether polyketide bearing a halogen-modified tetronate with neuroprotective and...
antiinvasive activity from Actinomadura sp. Org Lett. 2017; 19: 1406-1409.

5. Hasegawa T, Takizawa M and Tanida S. A rapid analysis for chemical grouping of aerobic actinomycetes. J Gen Appl Microbiol. 1983; 29: 379-372.

6. Hamada M, Yamamura H, Komukai C, Tamura T, Suzuki K and Hayakawa M. Luteimicrobium album sp. nov., a novel actinobacterium isolated from a lichen collected in Japan, and emended description of the genus Luteimicrobium. J Antimicrob Chemother. 2012; 67: 452-453.

7. Komaki H, Ichikawa N, Hosoyama A, Fujita N and Igarashi Y. Draft genome sequence of marine-derived Streptomyces sp. TP-A0598, a producer of anti-MRSA antibiotic lycidacmycins. Stand Genomic Sci. 2015; 10: 58.

8. Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H and Chun J. Introducing EzBioCloud: a taxonomically unified database of 165 rRNA gene sequences and whole-genome assemblies. Int System Evol Microbiol. 2017; 67: 1613-1617.

9. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ and Higgins GC. Clustal W and Clustal X version 2.0. Bioinformatics 2007; 23: 2947-2948.

10. Ohtsubo Y, Maruyasi F, Mitsuhi N, Nagata Y and Tsuda M. Complete genome sequence of Acidovorax sp. strain KKS102, a polychlorinated-biphenyl degrader. J Bacteriol. 2012; 194: 6970-6971.

11. Field D, Garrity G, Gray T, Morrison N, Selengut J, Sterk P, Tatusova T, Thomson N, Allen MJ, Angiuoli SV, Ashburner M, Axelrod N, Baldea S, Ballard S, Boeke J, Cochrane G, Cole J, Dowyanidt P, De Vos P, Defamphils C, Edwards R, Faruque N, Feldman R, Gilbert J, Gilna P, Gibson TJ and Higgins GC. Clustal W and Clustal X version 2.0. Bioinformatics 2007; 23: 2947-2948.

12. Hyatt D, Chen GL, Locascio PF, Land ML, Larimer FW and Hauser LJ. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 2010; 11: 19.

13. Lowe TM and Eddy SR. RfamScan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. 1997; 25: 953-964.

14. Weber T, Bli K, Duddela S, Dr K, HU Bruc Bli, Le Sy, Fischbach MA, Muller R, Wohlleben W, Breitling R, Takano E and Meirnas AH. antisSMASH 3.0.1a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res. 2015; 43: W237-243.

15. Meier-Kolthoff JP, Auch AF, Klenk HP and Goker M. Genome sequence-based species delineation with confidence intervals and improved distance functions. BMC Bioinformatics 2013; 14: 60.

16. W237-243.

17. Fischbach MA and Walsh CT. Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms. Chem Rev. 2006; 106: 3468-3496.

18. Sun Y, Zhou X, Dong H, Tu G, Wang M, Wang B and Deng Z. A complete gene cluster from Streptomyces nanchangensis SS226 encoding biosynthesis of the polyether ionophore nanchangmycin. Chem Biol. 2003; 10: 431-441.

19. Fang J, Zhang Y, Huang L, Jia X, Zhang Q, Zhang X, Tang G and Liu W. Cloning and characterization of the tetrocarcin A gene cluster from Micromonomospora chalcea NRRL 11289 reveals a highly conserved strategy for tetrone biosynthesis in spirotetronate antibiotics. J Bacteriol. 2008; 190: 6014-6025.

20. Gottardi EM, Kwak K, von Suchodoletz H, Schadt S, Muhlenweg A, Urgor CU, Pelser S, Fieider HP, Bibb MJ, Stach JE and Stussuad RD. Abyssomicin biosynthesis: formation of an unusual polyketide, antibiotic-feeding studies and genetic analysis. ChemBioChem 2011; 12: 1401-1410.

21. He HY, Pan HY, Wu LF, Zhang BB, Chai HB, Liu W and Tang GL. Quartromicin biosynthesis: two alternative polyketide chains produced by one polyketide synthase assembly line. Chem Biol. 2012; 19: 1313-1323.

22. Jia XY, Tian ZH, Shao L, Qu XD, Zhao QF, Tang J, Tang GL and Liu W. Genetic characterization of the chlorothricin gene cluster as a model for spirotetronate antibiotic biosynthesis. Chem Biol. 2006; 13: 575-585.

23. Sun Y, Hong H, Gillies F, Spencer JB and Leadlay PF. Glyceryl-S-acyl carrier protein as an intermediate in the biosynthesis of tetrone antibiotics. ChemBioChem 2008; 9: 150-156.

24. Saitou N and Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987; 4: 406-425.

25. Woese CR, Kandler O and Woese CR. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Nat Acad Sci USA. 1990; 87: 4567-4579.

26. Winter RE. Studies in the nomenclature and classification of the bacteria: II. The primary subdivisions of the Schizomycetes. J Bacteriol. 1917; 2: 155-164.

27. Skerman VBD, McGowan V and Sneath PHA. Approved lists of bacterial names. Int System Evol Microbiol. 1980; 30: 225-420.

28. Zhi XY, Li WJ and Stackebrandt E. An update of the structure and 16S rRNA gene sequence-based definition of higher ranks of the class Actinobacteria, with the proposal of two new suborders and four new families and emended descriptions of the existing higher taxa. Int System Evol Microbiol. 2009; 59: 589-606.

29. Zhang Z, Kudo T, Nakajima Y and Wang Y. Clarification of the relationship between the members of the family Thermomonosporaceae on the basis of 16S rDNA, 16S-23S rRNA internal transcribed spacer and 23S rRNA sequences and chemotaxonomic analyses. Int System Evol Microbiol. 2001; 51: 373-383.

30. Lechevalier HA, Lechevalier MP. A critical evaluation of the genera of aerobic actinomycetes. In The Actinomycetales (edited by Prauser H). Jenz Gustav Fischer Verlag, 1970: 95-405.

31. Fischbach MA, Ball CA, Blake JA, Botstein D, Butler H, cherry JM, Davis AP, Dolsinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Josel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM and Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 2000; 25: 25-29.