論文の内容の要旨

応用生命工学　　専攻

平成２５年度博士課程　進学

氏　　名　　トン　ウェイ リー

指導教員名　　西山　真

論文題目

Studies on novel polyketides discovered via activation of cryptic gene clusters in *Streptomyces*

（放線菌の潜在的遺伝子クラスターの活性化により発見した
新奇ポリケタイドに関する研究）

Actinomycetes, a group of Gram-positive bacteria with high G+C content, is well known as a prolific source of a wide range of bioactive secondary metabolites. The intense pursuit of bioactive metabolites from actinomycetes started back in the 1940s and early 1950s, when important antibiotics, such as the aminoglycosides, tetracyclines and macrolides, were discovered. To date, actinomycetes remain an important source for the production of naturally derived antibiotics that are used clinically. Despite the success of the microorganism as a source of natural products, conventional bioactive compounds screening have been deemed stagnant, and efforts are focused on the generation of synthetic compounds of their structural analogs instead. This is possibly due to the high rates of rediscovery of known compounds. In contrast, recent genome sequencing revealed that there are over 20 biosynthetic gene clusters (BGCs) in an actinomycete strain, predicted to encode different structural classes of secondary metabolites. Intriguingly, most of the predicted BGCs are classified as cryptic or orphan, because the metabolites produced by these cryptic BGCs are yet to be known. Hence, a growing number of efforts have been expended to develop methods that aim to unlock the cryptic pathways in actinomycetes.

While genome sequences have opened the doors for many successful cryptic gene activation approaches, I opted for a broader, sequence-independent strategy in this study. Here, the screening of mutants showing rifampicin resistance was applied to explore the potential novel compounds in *Streptomyces*. Spontaneous rifampicin-resistant (*rif*) mutants were reported to have mutation(s) in their *rpoB* gene (encoding RNA polymerase β-subunit) and the *rpoB* mutations are able to activate the production of bioactive compounds as demonstrated in the discovery of a cryptic new type of antibiotic called piperidamycin.

Chapter 1 Screening of rifampicin-resistant mutants and comparative metabolic analysis

The determination of minimum inhibitory concentrations (MIC) of rifampicin on 20 actinomycete strains used in this study showed that rifampicin was potent against most of the strains used at 3.125 μg mL^{-1} and below. *Streptomyces* sp. RM72 and *Streptomyces* sp. ND90, however, were found to have high MIC values of 25 μg mL^{-1} and 100 μg mL^{-1}, respectively, suggesting the possibility of natural resistance. The MICs obtained were then used as reference to choose the concentration of rifampicin needed for the generation of *rif* mutants in each strain. Spontaneous *rif* mutant generation in 20 actinomycete strains produced 278 mutants. Then, comparative metabolic analyses were performed on 200 *rif* mutants selected, along with their wild-type strains. Several improved production of metabolites by the *rif* mutants were detected in the screening. Among them, 4 overproduced metabolites (metabolites **1**, **3**, **6** and **7**) detected in the *rif* mutants (WK2057-10-39, TW-R50-13, S45-50-3 and S55-50-5), were selected for isolation and structural elucidation. The selection was based on the preliminary structure searches of these metabolites on SciFinder® and the Dictionary of Natural Products on DVD ver. 21:1, which suggested that they are potentially novel compounds.

Chapter 2 Identification of the overproduced secondary metabolites

Following the comparative metabolic screening in the chapter 1, isolations and structural elucidations were performed on the 4 metabolites **1**, **3**, **6** and **7** selected. Isolation of metabolite **1** from WK2057-10-39 resulted in 4 known phenoxazinones. On the other hand, purification from 6 L culture of TW-R50-13 yielded 2 novel compounds (compounds **3** and **3a**) with 2 distinct substituents, the 2-amino-3-hydroxycyclopent-2-enone (C_5N) and methylbenzene moieties (Figure 1). Besides that, metabolite **6** was elucidated as cinerubin A, while metabolite **7** was isolated as an unknown compound with a novel skeleton featuring an isoindolinone structure (Figure 1).

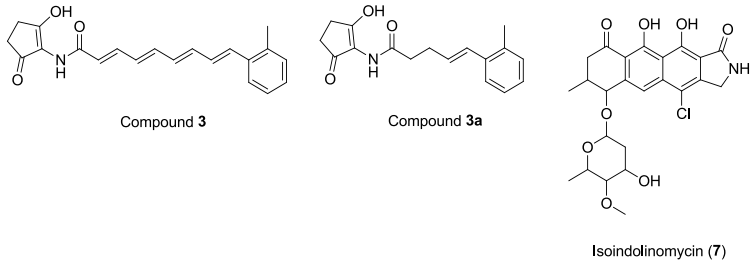

Figure 1. Novel compounds isolated in this study.

The novel compounds isolated were then evaluated for their biological activities. Compound **7** was found to show potent cytotoxicity against all cell lines tested (human ovarian adenocarcinoma SKOV-3, malignant pleural mesothelioma Meso-1, and T lymphoma Jurkat cells) and antimicrobial activity against *Staphylococcus aureus*. Compound **7** was then named as isoindolinomycin. On the contrary, no biological activity was detected

for compounds **3** and **3a** in all assays tested. Both *rif* mutants producing the novel compounds, TW-R50-13 and S55-50-5 have a point mutation in their *rpoB* gene at the position 437, which is a mutation point known to be effective in activating the production of secondary metabolites.

Chapter 3 Biosynthesis of the new compounds 3 and 3a isolated from the *rif* mutant TW-R50-13

Compounds **3** and **3a** are novel compounds featuring a C_5N unit and a methylbenzene moiety. Of these partial structures, the biosynthesis of the latter has not been reported. BLAST searches against the draft genome sequence of *Streptomyces* sp. SANK 60404 using a 5-aminolevulinate (ALA) synthase homologue, identified a BGC containing genes that may be related to the methylbenzene moiety (type I polyketide synthases) and C_5N unit (amide synthetase, ALA synthase, and acyl CoA ligase). The deletion of amide synthetase abolished the production of **3** and **3a**, confirming the relevance of the gene cluster. However, further analysis on the BGC showed that the deletion of the polyketide synthase (PKS) gene did not abolish the compounds, indicating that the genes are not responsible for the biosynthesis of **3** and **3a**. Because there is no other C_5N gene cluster found in the draft genome sequence of SANK 60404, I concluded that the BGC is only responsible for the C_5N moiety and that the genes for the methylbenzene moiety are located at a different locus. Then, a feeding experiment using ^{13}C-labeled precursors demonstrated that the methylbenzene moiety is most likely synthesized by the action of PKS. *In silico* screening on the draft genome sequence indicated that there are at least 7 BGCs encoding different types of PKS. Gene inactivation in 5 of these PKS gene clusters revealed that a BGC in Contig19 is involved in producing the methylbenzene moiety (Figure 2).

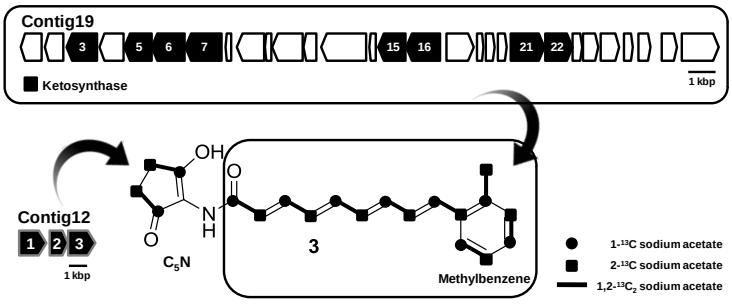

Figure 2. ^{13}C-labeling pattern and BGCs identified to synthesize compounds **3** and **3a**.

Unlike any other known PKSs, the gene cluster in Contig19 is made up of 8 discrete ketosynthases (KSs). A construction of phylogenetic tree showed that these KSs are categorized into 2 new clades distant from the other existing KSs. Further analysis on the gene cluster suggested that is it potentially a new type of PKS with unprecedented mechanism. The deletion of 4 genes in the BGC coupled with *in silico* analysis provided insight into the biosynthetic mechanism that produces the compounds **3** and **3a**.

Chapter 4 Biosynthesis of isoindolinomycin isolated from the *rif* mutant S55-50-5

To identify the BGC for isoindolinomycin, BLAST searches on the draft genome sequence of *Streptomyces* sp. SoC090715LN-16 was performed using a halogenase homologue as a query. The search returned two BGCs, each bearing a gene with close homology to tryptophan halogenase. Further analysis on the flanking regions showed that one of the BCG contains type II PKS, glycosyltransferase and methyltransferases, which are highly rational for the production of isoindolinomycin (Figure 3). The relevance of this BGC was then confirmed by gene disruption experiments. The biosynthetic studies of isoindolinomycin were mainly focused on 2 aspects: 1) the attachment of the methyl moiety to the core structure and 2) the formation of the 5-membered ring in the tetracyclic structure. For the first aspect, 3 methyltransferases (*idmB4*, *idmB24* and *idmB39*) found in the gene cluster were deleted to identify the gene responsible for the methyl moiety. The deletion of the methytransferases indicated that *idmB4* is the gene most likely responsible for the attachment of the methyl moiety to the tetracyclic structure, while *idmB39* may be involved in the *O*-methylation at the sugar moiety. Deletion of *idmB24*, however, did not cease the production of isoindolinomycin. In addition, no accumulation of intermediates was detected in the culture of *idmB4* deletion mutant, suggesting a possible unprecedented methylation by *idmB4* that occurs before the tetracyclic is formed. On the other hand, the formation of the 5-membered ring may involves the incorporation of an amino acid, presumably, glycine as the starter unit for the type II PKS system. To prove this assumption, a malachite green phosphate assay was performed to determine the substrate for the stand-alone adenylation domain (*idmB21*), which is known for its specific amino acid recognition activation as adenylyl amino acids. As expected, IdmB21 was found to display strict substrate specificity toward glycine only among the 20 amino acids tested. Taken together all the data obtained from *in silico* analysis, gene deletions and *in vitro* assay, the biosynthetic pathway for the unprecedented skeleton of isoindolinomycin was proposed.

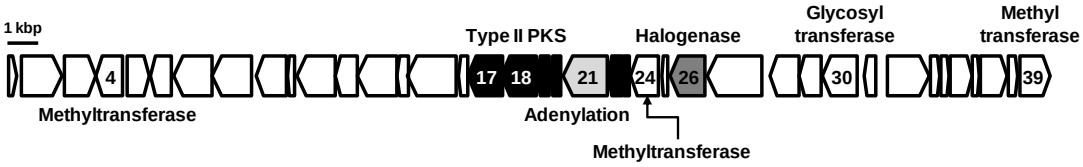

Figure 3. Organization of the BGC identified for isoindolinomycin.

Reference

Thong W. L, Shin-ya K, Nishiyama M, Kuzuyama T. Methylbenzene-containing polyketides from a *Streptomyces* that spontaneously acquired rifampicin resistance: Structural elucidation and biosynthesis. *J. Nat. Prod.* In revision.