Abstract of Doctoral Dissertation

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Studies on natural products containing nitrogen-nitrogen bond
(窒素-窒素結合を含む天然物に関する研究)

Rapid expansion of genomic information has revealed the abundant, yet unexploited biosynthetic potentials of natural products, such as those containing nitrogen-nitrogen (N–N) covalent bonds. Since the first discovery of macrozamin from *Macrozamia spiralis* in 1951, over 300 N–N bonds containing natural products had been dicovered.[1] Recent findings have revealed biosynthetic machineries for the biogenesis of N–N bond-containing functional groups,[2] which provided an opportunity for genome-mining approach for the discovery of N–N bonds-containing natural products. In this study, I focused on two biosynthetic machineries of N–N bonds containing functional groups: azoxy group and hydrazine group. For azoxy group, we conducted a reactivity-based screening targeting natural products containing azoxy moiety. For hydrazine group, we conducted global genome-mining approach by characterizing key N–N bonds forming enzymes *in vitro*.

Azoxy natural products are rare class of natural products with various beneficial biological properties, such as antibacterial, antifungal, nematicidal, and cytotoxic activities.[3] Biosynthesis of aliphatic azoxy natural products involves the *N*-hydroxylation of isobutylamine, mediated by the flavin-dependent monooxygenase *vlm*H, and the following formation of *O*-(L-seryl)-isobutylhydroxylamine by the tRNA-utilizing enzyme *vlmA*. Then this will be followed by intramolecular rearrangement to generate hydrazine structure accompanied by *vlmO*, and oxidation by *vlmB* to give azoxy moiety.[4–6] This implies that a genomic region containing these genes could be a potential biosynthetic gene cluster of aliphatic azoxy natural products. Therefore, we searched for potential biosynthetic gene cluster (BGC) of azoxy compounds which contain "*vlmA*", "*vlmH*", "*vlmO*", and "*vlmB*" genes from public database, and subjected them to network analysis tools called Bigscape[7]. As a result, these BGCs were classified into several clades and 96 strains from the network were chosen for further screening using *p*-dimethylaminobenzaldehyde (DAB), a method to detect the presence of hydrazine generated upon acid hydrolysis.[8] This facile and sensitive for detecting azoxy bonds has not been applied for natural products discovery. From this assay, positive results were detected from nineteen strains, and further consideration related to the novelty of BGC led us to choose *Streptomyces* sp. A1C6 for further isolation processes. As a result, azoxy compounds, isolated from culture metabolites in the reactivity-guided manner, were known aliphatic azoxy compounds azodyrecins A-C, and the geometric isomers, 1'-*trans* azodyrecins A-C, and azodyrecins E. Azodyrecins were previously isolated from several *Streptomyces* strains including *Streptomyces* sp. RM72. Comparison of gene clusters for azodyrecins (*ady*) from strain RM72 and A1C6 identified a similar yet distinct type of azodyrecin biosynthetic gene cluster that contains several insertions and inversions. Taken together, the N_2H_4-detecting reactivity-based screening identified a new type of biosynthetic gene clusters of azodyrecins, demonstrating its utility in natural product discovery and deorphanization of biosynthetic gene clusters.[9] Furthermore, azodyrecin B and 1'-*trans*-azodyrecin B showed potent cytotoxic activity against SKOV3, MESO1, Jurkat cell (IC_{50} 7,37-9,7 mM for azodyrecin B; IC_{50} 3,36-8,24 mM for 1'-*trans*-azodyrecin B, while the saturated analogs azodyrecin D and E exhibited no cytotoxicities. These results indicate that the double bond adjacent to the azoxy moiety plays an important role for the biological activity of azodyrecins.

In the second part, I did exploration of N-N bond forming enzyme in bacteria. Hydrazine synthetases (HSs) catalyze the N–N bond formation between canonical amino acids and hydroxylamines, which are provided by cognate flavin-dependent *N*-hydroxylase (NMO)[10,] and the resultant hydrazines are

utilized as key precursors of N–N bonds-containing natural products. The structure of hydrazine can be divided into two parts: a hydroxylamine-derived part and an amino acid-derived part. Bioinformatic analysis indicates that the HSs characterized to date represent only a small portion of the entire sequence diversity of this protein family[11]. Moreover, HSs are distributed across several bacterial phyla, while most of the experimentally characterized ones are only limited to those derived from actinobacteria. These observations suggest that the chemical diversity of the hydrazines generated by HSs have not been fully explored. In this study, I conducted global genome-mining of hydrazine-forming pathways. First, I retrieved 422 NMOs involved in hydrazine biosynthesis from public database. Phylogenetic analysis showed that these enzymes are categorized into seven groups, which presumably possess distinct substrate specificities. To elucidate these, genes for representative NMOs were synthesized and recombinant NMOs were expressed in *E. coli*. *In vitro* analysis revealed substrate amino acids for each group of NMO, including DAP, DABA, ornithine, and lysine. NMOs in several minor groups were inactive against amino acids tested, suggesting that they catalyze *N*-hydroxylation against specialized amines that are biosynthesized by enzymes encoded in the neighboring genomic regions. Overall, this survey gave a global view for the functional diversity of NMOs in hydrazine biosynthesis. Next, 401 HSs were retrieved from public databases and subjected to phylogenetic analysis. HSs were classified into more than nine groups which presumably possess distinct substrate specificities. To elucidate the hydrazine products, representative HSs were expressed in *E. coli* host. They were cultured in M9 media and supplemented with N-OH amino acids, then overnight grown culture broth was subjected to LC-MS analysis. This survey successfully identified the new hydrazine products derived from three HSs system; DABA-glu, ornithine-tyrosine, DABA-serine. Notably, DABA-glu was the first hydrazine product derived from *Firmicutes*.

Overall, the detailed phylogenetic analysis of NMOs and HSs provided a global view on the functional diversity of this N–N bonds-forming machinery. Regarding their wide occurrence and diversity, the gene cassettes of NMO and HS could be regarded as one of the signatures for BGCs of N–N bonds-containing natural products.

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