Nocardamin Production by *Streptomyces avermitilis*

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Desferrioxamine B (desferal®) is the only therapeutic agent for chronic iron overload and acute iron intoxication. Therefore, new drug candidates are required for more effective treatments. Recent genome analyses of microorganisms have enabled us to identify cryptic gene clusters of secondary metabolism. In a genomic analysis of *Streptomyces avermitilis*, we found the putative biosynthetic gene cluster for nocardamin and desferrooxamine derivatives, the production of which by *S. avermitilis* had been unknown. To determine the synthesis of nocardamin and discover new derivatives in *S. avermitilis*, we performed a comprehensive analysis and isolation of secondary metabolites of *S. avermitilis*. We obtained nocardamin and its related compounds, including a derivative that initially was isolated as a microbial product. The biosynthetic pathway of nocardamin and the substrate specificity of its biosynthesis enzymes were proposed based on the structures of the isolated nocardamin derivatives. The production of nocardamin by *S. avermitilis* was completely suppressed by the addition of more than 5 μM ferric ions. An iron-dependent regulatory protein (IdeR)-binding motif was located upstream of the *sidABCD* operon, using the profile hidden Markov model method. To determine the regulation of nocardamin synthesis by IdeR in *S. avermitilis*, we constructed *ideR* deletion mutants. The production of nocardamin by the deletion mutants was not suppressed by ferric ions (up to 100 μM).

Introduction

Nearly all microorganisms require iron as an essential element for growth, acquiring it from their environment using small-molecule compounds called siderophores as high-affinity ferric ion chelators. Siderophores are primarily divided into 3 classes—catecholates, hydroxamates, and α-hydroxycaboxylates—depending on the chemical nature of the moieties that donate the oxygen ligands for ferric coordination (Michthe & Marahiel, 2007).

Nocardamin (1), a hydroxamate siderophore, was initially isolated as an antibacterial metabolite of a *Nocardia* strain (Stoll, *et al.*, 1951). Several decades later, ferroxamine derivatives were isolated from several actinomycetes (Hossan *et al.*, 1983). The structure of ferrioximane E (3) is essentially identical to that of 1, but 3 chelates a ferric ion. Thus, 1 is known as desferrooxamine E, which is 3 that has lost its ferric ion.

Various bacteria produce 1 and related siderophores to take up ferrous and ferric ions to compete with other bacteria. Several pathogenic bacteria that have lost the ability to synthesize siderophores are less pathogenic (Ojha & Hatfull, 2007). Therefore, we are interested in understanding how the production of nocardamin and its related siderophores is controlled.

Many genomic analyses of microorganisms have been performed in the last 10 years. A genomic analysis of *Streptomyces avermitilis*, which produces the antihelminthic macrocyclic lactone avermectin, has been completed (Ikeda *et al.*, 2003), yielding many gene clusters of secondary metabolism. One of them, a gene cluster that comprises 6 genes, was proposed to function in the biosynthesis of nocardamin or its related metabolites. In this report, we describe the purification and identification of siderophores from *S. avermitilis* and identify the mechanisms that regulate the production of nocardamin in *S. avermitilis*.

Materials and methods

Isolation and identification of nocardamins

*S. avermitilis* K139 (isogenic strain of *S. avermitilis* MA-4680, ATCC 31267, NRRL 8165, and JCM 5070) was cultured at 28°C for 5 days in production medium containing 2% glucose, 1% soluble starch, 0.3% meat extract, 2.5% dried yeast, 0.3% corn steep liquor, 0.05% K₂HPO₄, 0.05% NaCl, 0.05% CaCO₃, and 0.05% MgSO₄·7H₂O and adjusted pH to 6.0 with 1N NaOH before autoclaving. Five liters of cultured broth was extracted with an equal volume of acetone and extracted twice with an equal volume of n-butanol after removal of acetone in vacuo. After successive separations of the butanol extract with HPLC using ODS columns, nocardamin derivatives were purified. Identification and structural elucidation of the purified compounds were performed in NMR experiments and by mass spectrometry.

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Deletion of ideR (SAV3855)

Deletion of the ideR gene was performed as described (Tetzlaff, 2006). Two fragments, corresponding to the upstream and downstream regions of ideR, were amplified by PCR from the cosmid CL220H01 using the following pairs of primers—up-forward (5'-CTCGAGGAATTC-AGTGAAGTGCCACCCAGATC-3') and up-reverse (5'-CTCGAGAAATTCATTGCTGCCCTCGAT-3') for the upstream region, and down-forward (5'-CTCGAGAAGCTTCAGGTTGACCTGCGG-3') and down-reverse (5'-CTCGAGAAATTCCATGCTAACCCAGATC-3') for the downstream region—to introduce EcoRI and HindIII restriction sites (in bold), respectively.

After digestion of the amplified fragments by EcoRI and HindIII, traces of the amount DNA were removed by DpnI digestion. The two amplified fragments that were digested with EcoRI and HindIII and the 1.2-kb HindIII fragment that carried aphII, which was prepared by PCR using the forward (5'-GAAGCTTCTTCTACGCCTCGC-3') and reverse (5'-GAAGCTTCTCTACGCCTCGC-3') primers (the bold characters indicate HindIII sites), respectively. A solution of FeCl$_3$ was added to reach the concentration of 600 μM. Two fragments that were prepared directly from soil (Wang et al., 2000), 1.4 mg of desferrioxamine D1 (5), 0.9 mg of N-butryldesferrioxamine B (6), 1.3 mg of desferrioxamine A1 (7). The structures of some derivatives could not be determined due to their small quantity and low stability.

Nocardamin derivatives from S. avermitilis.

Based on the genomic analysis of S. avermitilis, we speculated on the biosynthesis of nocardamin-related compounds. The actual production by this strain, however, was unknown until nocardamin and its derivatives were identified from the culture broth, as described in this report. To isolate nocardamin and its related metabolites, we performed UV-guided fractionation by HPLC, although the metabolites did not exhibit typical UV absorption, except for absorption at 220 nm by peptide bonds.

Although the siderophores produced by S. avermitilis were composed primarily of nocardamin and ferrioxamine E (ferric ion-chelating nocardamin), a detailed analysis and isolation of the metabolites allowed us to identify the minor components of siderophores. From a 5-liter culture, we obtained 5.6 mg of nocardamin (1), 4.2 mg of terragine E (2), 1.4 mg of ferrioxamine E (3), 10 mg of N-butyrylferrioxamine B (4), 0.6 mg of desferrioxamine D1 (5), 0.9 mg of N-butrylferrioxamine B (6), 1.3 mg of desferrioxamine A1 (7). The structures of some derivatives could not be determined due to their small quantity and low stability.

1H and 13C NMR data of nocardamin (1) revealed an N-hydroxy-N-succinylcadaverine unit, whose molecular weight corresponded to 200, while FABMS analysis of 1 generated a peak at m/z 601 ([M+H]+). Therefore, 1 consisted of three N-hydroxy-N-succinylcadaverine units that were conjugated iteratively.

Nocardamin biosynthesis in S. avermitilis during iron starvation and quantification of nocardamin.

S. avermitilis K139 was cultured at 28°C for 5 days in a 100-ml Erlemeyer flask containing 10 ml of medium (2.0% dextrin, 2.0% mannitol, 0.05% K$_2$HPO$_4$, 0.025% MgSO$_4$·7H$_2$O, 0.005% ZnSO$_4$·7H$_2$O, 0.05% CaCO$_3$, 0.12% L-asparagine, 0.025% L-lysine, 0.01% L-methionine, and 0.01% L-threonine, adjusted pH to 7.0 before sterilization). A solution of FeCl$_3$ was added to reach the appropriate concentrations of ferric iron prior to inoculation. The culture broth was extracted with an equal volume of n-butanol, and n-butanol aliquots were concentrated in vacuo. The butanol extracts from the 10-ml culture were dissolved in 200 μl methanol, and 5 μl of the extract was directly analyzed by HPLC or LC-MS.
Desferrioxamine B (6) was chemically synthesized to compare the solution equilibria with other derivatives under various conditions (Ihnat et al., 2000), no report has identified 6 as a microbial metabolite. Thus, this study is the first to isolate it as a natural product.

Desferrioxamine B (4) is an important microbial metabolite for clinical use, because it is the only drug that treats chronic iron overload (hemochromatosis) and acute iron intoxication. The hydrophilic nature of 4 has already been characterized well by the low distribution coefficient ($P_{\text{octanol/water}} = 0.0013 \pm 0.0003$) and the high aqueous solubility ($>38.0 \pm 4.4 \text{ g/100 mL}$) in the pH range of 4.2 to 8.0 at $25^\circ C$ (Ihnat et al., 2000). Consequently, many derivatives have been synthesized and characterized. However, the physical properties of 4 are now seemed to be the most suitable for the clinical usage in various known or considerable derivatives of the hydroxamate siderophores.

Before we began the studies on nocardamin production by *S. avermitilis*, we have performed a comprehensive analysis of metabolites in the broth extracts cultivated in twenty different media using LC/MS. During this metabolite analysis, we detected a probable nocardamin signal ($m/z$ 601) and its fragmented signals ($m/z$ 401 and 201) in several MS charts. One of media was selected for the production of nocardamin because a lot of metabolites were detected in the extract of the selected medium. Although the structures of several nocardamin derivatives could not be elucidated in this study, they showed unique mass spectra and $^1H$ NMR signals that have not been reported. The comprehensive analysis and isolation of microbial metabolites often yield new metabolites. Thus, microorganisms produce myriad metabolites that we cannot predict or create.

**Proposed biosynthetic pathway of nocardamins in *S. avermitilis***.

The biosynthetic cluster for siderophores in *S. avermitilis* is composed of 6 genes, *sidA* $\sim$ *sidF* (Fig. 2A), and we propose a biosynthetic pathway, as shown in Fig. 3, according to the proposed functions of each gene product. Lysine decarboxylase, encoded by *sidD*, removes a carboxylate moiety from L-lysine to yield cadaverine. Cadaverine is hydroxylated at an amino group by SidC, *cadaverine N*-monooxygenase, to form *N*-hydroxycadaverine (HC). Subsequently, SidB, a putative succinyl-CoA transferase, is thought to condense succinyl-CoA and cadaverine or HC to *N*-succinylcadaverine (SC) and *N*-hydroxy-*N*-succinylcadaverine (HSC), respectively. SC and HSC are the components for siderophore biosynthesis in several microorganisms, not merely in *S. avermitilis*.

In *S. avermitilis*, the gene product of *sidA*, appears to catalyze the assembly of three HSC and/or SC units to form nocardamin and its derivatives. The substrate specificity of SidB is likely to be promiscuous, because SidB occasionally uses other acyl-CoA units as substrate to form *N*-acyl derivatives of cadaverine. When acetyl-CoA is used by SidB to form *N*-hydroxy-*N*-acetylcadaverine (HAC), SidA can assemble HAC and SC (HSC) to form an acyclic siderophore (4). Desferrioxamine B (4) is often produced.
by many eubacteria. The other two genes, sidE and sidF, regulate the incorporation of extracellular iron-siderophore complexes, because their gene products are similar to siderophore-interacting protein and ABC transporter, respectively.

The structures of nocardamin derivatives contain amide bonds, which can be classified into two types, according to their mode of assembly. One amide bond is an essential building block, formed by acyl-CoA-mediated acyltransferation. Genes encoding enzymes that mediate this amide bond formation are found in other gene clusters of siderophore biosynthesis in various microorganisms, and they are homologous to acyl-CoA acyltransferases. Another amide bond is formed when building blocks are assembled by the siderophore synthase SidA and its homolog proteins, which belong to the IucA/IucC family (Martinez et al., 1994). Genes encoding IucA/IucC family proteins are also observed as siderophore syntheses in biosynthetic gene clusters in many eubacteria, but their actual function and reaction mechanisms had been unknown for long time. Recently, G. L. Challis and colleagues reported ATP-dependent oligomerization and macrocyclization of the building blocks using DesD from S. coelicolor M145 (Kadi et al., 2007) and PusC from Shewanella putrefaciens (Kadi et al., 2008). The siderophore synthase responsible for this terminal step is unique to eubacteria and is absent in eukaryotic cells. The siderophore synthase appears to be suitable as an antibacterial target protein, because the virulence of pathogenic bacteria is closely related to their production of siderophores (Miethke & Marahiel, 2007).

Regulation of nocardamin biosynthesis by iron and regulatory protein ideR.

Most organisms require iron as an essential element for many metabolic and cellular signal pathways. To take up iron from the environment, where it is insoluble (Fe(OH)$_3$; $<2 \times 10^{-9}$ M) under aerobic conditions, microorganisms secrete iron-chelating siderophores, which solubilize insoluble iron, and incorporate iron-siderophore complexes.

Bacteria that have a high GC content, such as streptomycetes, corynebacteria, and mycobacteria, express an iron-dependent repressor protein, named DtxR, after the first member of the protein family that was characterized as an iron-dependent diphtheria toxin regulator. These DtxR-like proteins regulate many genes that are similar to those that are regulated by Fur proteins in Gram-negative bacteria, ranging from siderophore biosynthesis and iron uptake genes to oxidative stress response genes (Ratledge & Dover, 2000; Hantke, 2001). A dtxR ortholog, ideR (SAV3855), has been annotated in S. avermitilis, and a putative binding sequence (Tao et al., 1994; Gold et al., 2001) of IdeR was observed upstream of the sidABCD and sidEF operons, using the profile hidden Markov model method (Fig. 2B; Rabiner, 1989).
To determine whether IdeR regulates expression of the *sidABCD* operon, we confirmed the production of nocardamins in an *ideR*-disrupted mutant. We did not observe any difference in morphological differentiation or growth rate between the wild-type strain and the *ideR*-disrupted mutants. An *ideR* disruptant (∆*ideR*) produced more nocardamins compared with the wild-type strain. The production decreased slightly at increased iron concentrations, but the production still remained under higher concentrations of iron (up to 100 μM). In the wild-type strain, the production of nocardamins was completely suppressed at 5 μM Fe³⁺ (Fig. 4). The reduced production at high Fe³⁺ concentrations in the *ideR* disruptant was affected by another iron-dependent element(s), because *S. avermitilis* harbors the putative orthologs of furA (SAV4029) and furB (SAV5631).

The transcriptional regulator DtxR and its ortholog IdeR regulate the control of iron metabolism in many microorganisms (mainly Gram-positive bacteria). In the presence of iron, however, these regulators suppress and induce the expression of several genes. In *Corynebacterium glutamicum*, DtxR affects the expression levels of 34 operons coding 64 genes, which are involved in many metabolic processes, such as iron acquisition, transcriptional regulation, and fatty acid synthesis (Wennerhold & Bott, 2006). Presumably, *ideR* disruption also affects the expression of many genes (about 24 candidate genes were found by profile hidden Markov models analysis) in *S. avermitilis*, nearly all of which control the metabolism of iron and siderophores. To clearly demonstrate that IdeR regulates the expression of the *sidABCD* operon, other experiments are needed.

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**Fig. 3.** Proposed biosynthetic pathway of nocardamin and desferrioxamine derivatives.

The biosynthetic pathway of nocardamin and desferrioxamine derivatives is proposed, based on the predicted functions of biosynthetic genes in *S. avermitilis* K139.

**Fig. 4.** Nocardamin production in wild-type and *ideR* disruptant.

Nocardamin production by wild-type (open circle) was completely repressed by more than 5 μM of ferric ion in the culture medium. Production by the *ideR* disruptant (closed circle) decreased slightly but was not suppressed at higher concentrations of iron (up to 100 μM).
Conclusion

In this study, we purified nocardamin and desferrioxamine derivatives from the culture broth of *S. avermitilis* K139, which is expected to produce nocardamin, based on our genomic analysis. Although various analogs of desferrioxamine have been synthesized chemically and characterized, there is no useful synthetic derivative other than desferrioxamine B (4, known as desferal9 in clinical use) which is currently the only available therapeutic agent for chronic iron overload and acute iron intoxication. Therefore, new drug candidates are required for more effective treatments, because the structures of natural products often exhibit the ideas beyond our thought. So we have to keep learning the nature ideas hidden in the microbial metabolites. This is the aim itself of microbial natural product research.

We began a comprehensive analysis of metabolites to identify new derivatives of desferrioxamine from *S. avermitilis* K139, and we ultimately obtained nocardamin and 6 desferrioxamine derivatives, including a compound that was determined to be a new microbial metabolite. *S. avermitilis* K139 produces many polyketide metabolites, such as avermectins, filipins, and oligomycin, so it is difficult to discover minor metabolites. Minor secondary metabolites, however, can be preferentially expressed under suboptimal production conditions. In this work, we analyzed the extracts of *S. avermitilis* K139 that were prepared from culture broths of twenty different media, and one of twenty culture media was selected for nocardamin derivative production and isolation. But, it remained unknown what factor(s) containing in culture medium affected the production of secondary metabolites, such as nocardamin and its derivatives. Although several “ome” analyses might be required to clarify the detailed regulatory mechanism of secondary metabolite production, we finally conclude that the comprehensive analysis and isolation of secondary metabolites can be the strong strategy to identify the potential capacities for metabolism and production hidden in the genome.

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