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ARTICLE

Novel desferrioxamine derivatives synthesized using the secondary metabolism-specific nitrous acid biosynthetic pathway in *Streptomyces davawensis*

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Abstract

Recently, a novel nitrous acid biosynthetic pathway composed of two enzymes was discovered to be involved in the biosynthesis of cremeomycin for the formation of its diazo group. In this pathway, CreE oxidizes L-aspartic acid to nitrosuccinic acid and CreD liberates nitrous acid from nitrosuccinic acid. Bioinformatic analysis showed that various actinobacteria have putative secondary metabolite biosynthesis gene clusters containing *creE* and *creD* homologs, suggesting that this pathway is widely used for the biosynthesis of various natural products. Here, we focused on *creE* and *creD* homologs (*BN159_4422* and *BN159_4421*) in *Streptomyces davawensis*. In vitro analysis of recombinant BN159_4422 and BN159_4421 proteins showed that these enzymes synthesized nitrous acid from L-aspartic acid. Secondary metabolites produced by this gene cluster were investigated by comparing the metabolic profiles of the wild-type and Δ*BN159_4422* strains. When these strains were co-cultured with *Tsukamurella pulmonis* TP-B0596, three compounds were specifically produced by the wild-type strain. These compounds were identified as novel desferrioxamine derivatives containing either of two unique five-membered heterocyclic ring structures and shown to have iron-binding properties. A putative desferrioxamine biosynthetic gene cluster was found in the *S. davawensis* genome, and inactivation of a *desD* homolog (*BN159_5485*) also abolished the production of these compounds. We propose that these compounds should be synthesized by the modification of desferrioxamine B and a shorter chain analog using nitrous acid produced by the CreE and CreD homologs. This study provides an important insight into the diverse usage of the secondary metabolism-specific nitrous acid biosynthetic pathway in actinomycetes.

Introduction

Actinomycetes have been a useful source of bioactive natural products [1]. For instance, the immunosuppressant FK506 and the antibiotic vancomycin were discovered from this class of microorganisms. However, the discovery rate of natural products with novel structures is decreasing [2], and some previously discovered compounds are often found repeatedly, although novel antibiotics are still needed because of the emergence of multidrug-resistant pathogens. In contrast, the recent advances in next-generation DNA sequencers have resulted in extensive genomic information on actinomycetes and showed that most of these microorganisms have at least 30 secondary metabolite biosynthesis gene clusters [3]. This number is much higher than the numbers of natural products that have been isolated from respective strains, suggesting that most of such biosynthetic gene clusters are not active under usual laboratory conditions. These silent gene clusters are expected to be a useful source of novel natural products.

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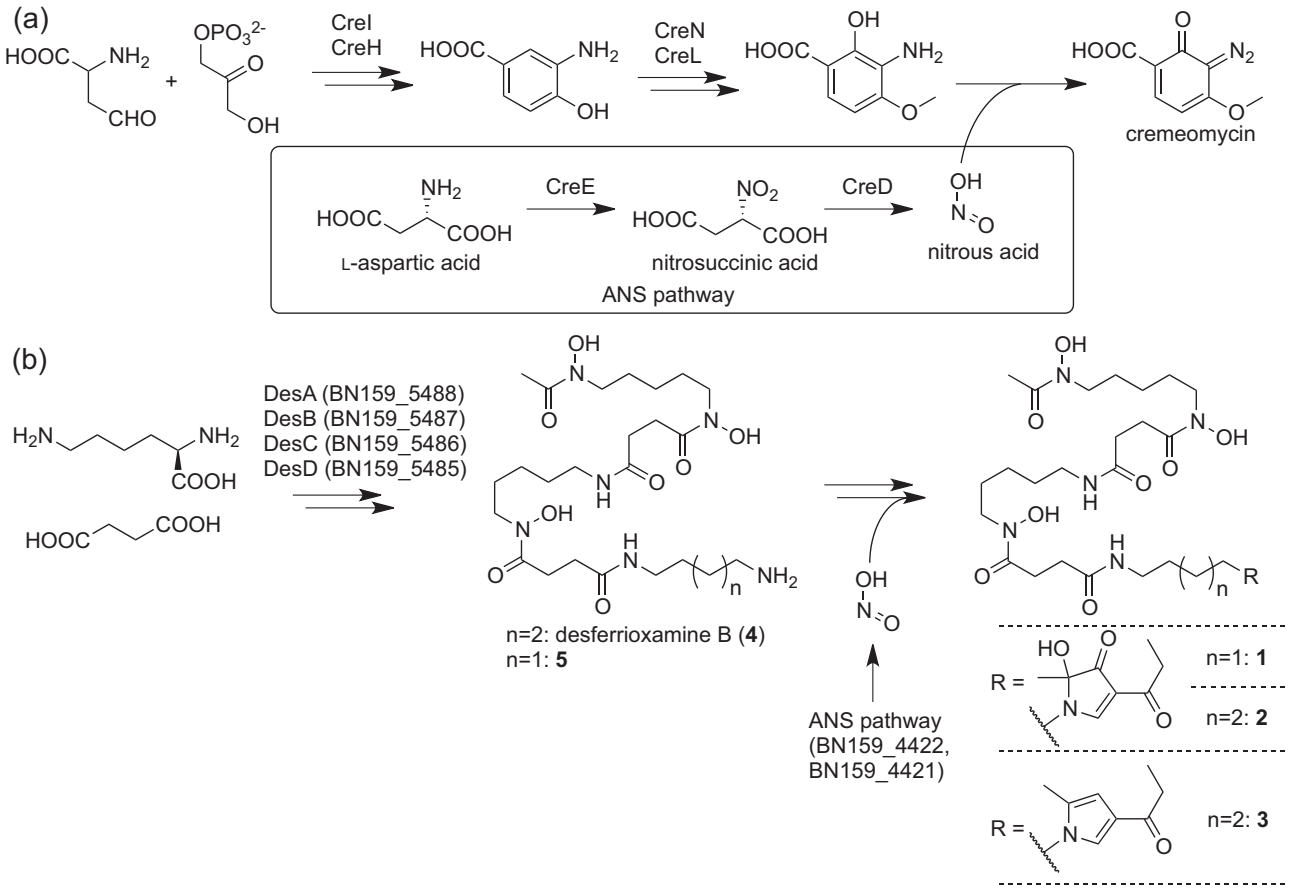

Fig. 1 Cremeomycin biosynthetic pathway (**a**) and putative biosynthetic pathway for novel desferrioxamine derivatives (**b**)

Genome mining is an approach to discover novel natural products based on genetic information [3]. The activation of silent gene clusters is one of the most important parts of genome mining. Therefore, many methods have been developed to activate silent biosynthetic gene clusters [3–6]. One approach is to cultivate a certain microorganism with another microorganism. This co-culture method often results in the production of natural products that are not produced in pure culture. Co-cultivation of *Streptomyces* with a mycolic acid-containing bacterium, for example, is called combined-culture, and various compounds have been isolated using this method [4, 7–12].

Recently, we analyzed the biosynthetic pathway of cremeomycin and discovered an unprecedented nitrous acid biosynthetic pathway composed of two enzymes, CreE and CreD [13]. Nitrous acid produced by this pathway is involved in the formation of the diazo group of cremeomycin (Fig. 1a). In this pathway, first CreE converts L-aspartic acid to nitrosuccinic acid. Sequentially, CreD converts nitrosuccinic acid to nitrous acid and fumaric acid. The nitrous acid reacts with the amino group of 3-amino-2-hydroxy-4-methoxybenzoic acid to form the diazo group of cremeomycin. We call this the ANS (L-aspartate-nitrosuccinate) pathway in this manuscript. In silico analysis of genome sequences in the public databases showed that operons consisting of *creE* and *creD* homologs are present in a wide variety of actinomycetes [13]. In addition, many of these operons are surrounded by genes apparently related to secondary metabolism, suggesting that the ANS pathway is involved in the biosynthesis of various secondary metabolites. This idea was supported by a study on the fosfazinomycin biosynthetic pathway [14]. FtzM and FtzL, encoded by the fosfazinomycin biosynthetic gene cluster, have the same function as CreE and CreD and are presumably involved in the biosynthesis of the N–N bond of fosfazinomycin. However, we have still limited knowledge of the role of the ANS pathway in secondary metabolism.

To further understand this role, we carried out genome mining of the ANS pathway. We selected a putative secondary metabolite biosynthetic gene cluster containing *creE* and *creD* homologs in the genome of *Streptomyces davawensis* JCM 4913 as a target (Fig. 2 and Table 1). By comparing the metabolic profiles of the wild-type and Δ*creE* (*BN159_4422*) strains, we identified three novel desferrioxamine derivatives having either of two unusual five-membered ring structures (Fig. 1b). This research expands our knowledge on the role of the ANS pathway for the biosynthesis of secondary metabolites.

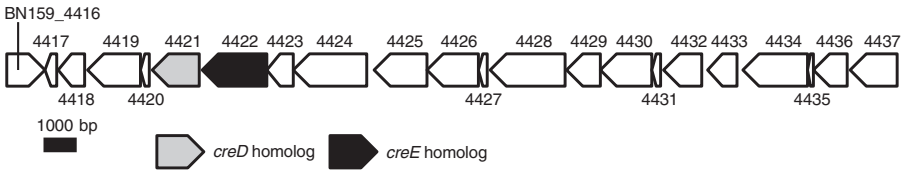

Fig. 2 Biosynthetic gene cluster containing *creE* and *creD* homologs in *S. davawensis*

Table 1 Predicted functions of the genes located around the *creE* and *creD* homologs

Gene id	aa length	Putative function
BN159_4416	377	Acyl-CoA dehydrogenase
BN159_4417	115	Rhodonase-like domain-containing protein
BN159_4418	266	DnaJ-like domain-containing protein
BN159_4419	519	Hypothetical protein
BN159_4420	91	Hypothetical protein
BN159_4421	474	CreD (nitrosuccinate lyase) homolog
BN159_4422	650	CreE (flavin-dependent monooxygenase) homolog
BN159_4423	273	4′-phosphopantetheinyl transferase
BN159_4424	737	TrpE(G)-like protein
BN159_4425	536	Flavin-dependent monooxygenase
BN159_4426	515	CoA-ligase
BN159_4427	85	Acyl carrier protein
BN159_4428	750	Membrane protein
BN159_4429	332	Transglutaminase
BN159_4430	521	AMP-binding enzyme
BN159_4431	93	Acyl carrier protein
BN159_4432	382	NAD-dependent epimerase/dehydratase
BN159_4433	298	Alpha/beta hydrolase
BN159_4434	646	Transcriptional regulator
BN159_4435	67	Hypothetical protein
BN159_4436	331	AraC family transcriptional regulator
BN159_4437	462	Class-II DAHP synthetase

Materials and methods

Strains and chemicals

S. davawensis JCM 4913 was obtained from the Japan Collection of Microorganisms (JCM, Ibaraki, Japan). *T. pulmonis* TP-B0596 was isolated from the soil sample from Toyama Prefecture, Japan, previously [15]. *Escherichia coli* JM109 was used for DNA manipulation. *E. coli* ET12567 harboring pUZ8002 was used for conjugation. TSB medium was prepared by dissolving 3% tryptic soy broth in water and autoclaving prior to incubation. A-3M medium was prepared by dissolving 0.5% glucose, 2% soluble starch, 2% glycerol, 1.5% Pharmamedia®, 0.3% yeast extract, and 1% HP-20 (Mitsubishi Chemical Corporation, Tokyo, Japan), adjusted to pH 7.0, and autoclaving prior to incubation. Bennett maltose medium was prepared by dissolving 0.1% yeast extract, 0.07% bonito extract, 0.038% meat extract, 0.2% NZ amine type A, and 1% maltose, adjusting to pH 7.3, and autoclaving prior to incubation. Restriction enzymes and DNA polymerase were purchased from Takara Bio Inc. (Shiga, Japan). In the polymerase chain reaction (PCR), the genomic DNA of *S. davawensis* was used as a template unless otherwise noted.

Production and purification of BN159_4422 (CreE homolog) and BN159_4421 (CreD homolog)

BN159_4422 was amplified by PCR using primers 5′-CAT*ATG*ACCGGCAGCAGGACCAA-3′ (an NdeI site is underlined, and the start codon is italicized) and 5′-AAGCTTTACGATCACCCCGGCGCCCT-3′ (a HindIII site is underlined, and the stop codon was removed for the histidine-tag fusion at the C-terminus of the recombinant protein). The obtained DNA fragment was cloned into NdeI and HindIII sites of pET26b, resulting in pET26-BN159_4422. *BN159_4421* was amplified by PCR using primers 5′-CAT*ATG*AGCGGGCGAGGCGACAC-3′ (an NdeI site is underlined, and the start codon is italicized) and 5′-AAGCTT*TCA*GCGCAGGGCGCGGTCCA-3′ (a HindIII site is underlined, and the stop codon is italicized). The obtained DNA fragment was cloned into the NdeI and HindIII sites of pColdI, resulting in pColdI-BN159_4421.

E. coli BL21(DE3) strains harboring pET26-BN159_4422 and pColdI-BN159_4421 were individually cultured in 100 mL of TB medium with 50 μg/mL kanamycin at 37 °C until OD_{600} reached 0.6. After incubation at 18 °C (for the strain harboring pET26-BN159_4422) and 15 °C (for the strain harboring pColdI-BN159_4421) for 15 min, 0.05 mM isopropyl β-D-L-thiogalactopyranoside (IPTG) with 2 mg/L riboflavin and 0.02 mM IPTG, respectively, was added into the culture broths. After 20 h, the cells were harvested using centrifugation and resuspended in lysis buffer I (20 mM Tris-HCl pH 9.0, 20% glycerol, 200 mM NaCl, 10 mM β-mercaptoethanol) and lysis buffer II (20 mM HEPES pH 8.0, 20% glycerol, 500 mM NaCl), respectively. Then lysozyme (final ~0.5 mg/mL) was added to each solution. After each solution was incubated on ice for 1 h, the cells were lysed using sonication [0.05 mM flavin adenine dinucleotide (FAD) was added to the sample of the strain harboring pET26-BN159_4422 prior to sonication]. After cell debris was removed by centrifugation, the recombinant BN159_4422 and BN159_4421 proteins were purified by Ni^{2+} affinity

chromatography using His60 Ni Superflow Resin (Takara Bio Inc.; 1 mL). The former and latter proteins were eluted with elution buffer I (20 mM Tris-HCl pH 9.0, 20% glycerol, 200 mM NaCl, 10 mM β-mercaptoethanol, 200 mM imidazole) and elution buffer II (20 mM HEPES pH 8.0, 20% glycerol, 500 mM NaCl, 200 mM imidazole), respectively, desalted with the same lysis buffer and concentrated using an Amicon Ultra centrifugal filter with a 10,000-molecular-mass cutoff (Merck Millipore, Darmstadt, Germany).

In vitro assay of CreE and CreD

The reaction mixture containing 10 μM CreE, 10 μM CreD, 5 mM L-aspartic acid, 2 mM NADPH, and 100 mM Tris-HCl (pH 7.5) was incubated at 30 °C for 1 h. The reaction was quenched by adding formic acid (final concentration 2%). Production of nitrous acid was examined with Saltzman reagent as described previously. [16]

Construction of the Δ*BN159_4422* (*creE* homolog) strain

The *BN159_4422* disruptant was constructed by substituting the core region of *BN159_4422* with an apramycin resistance gene (*aac(3)IV*) by homologous recombination. A downstream fragment (~2000 bp) of *BN159_4422* was amplified by PCR using primers 5′-AAGCGGCCGCTGCTGTCTGGTGGTCATGTC-3′ (a NotI site is underlined) and 5′-TTGATATCATCCGTACGCTGCTCAACTC-3′ (an EcoRV site is underlined). The obtained DNA fragment was cloned into NotI and EcoRV sites of pKGLP2 [17], resulting in pKGLP2-BN159_4422-down. Meanwhile, *aac(3)IV* was amplified by PCR using primers 5′-AATCTAGAAGCAAAAGGGGATGATAAGTTTATC-3′ (an XbaI site is underlined) and 5′-TTGCGGCCGCAGAATAGGAACTTCGGAATAGG-3′ (an NotI site is underlined) and an *acc(3)IV*-containing DNA fragment as a template. The obtained DNA fragment was cloned into XbaI and NotI sites of pKGLP2-BN159_4422-down, resulting in pKGLP2-BN159_4422-down/apra. An upstream fragment (~2000 bp) of *BN159_4422* was amplified by PCR using primers 5′-AACTGCAGCATCAACTCGTGCCGACGGT-3′ (a PstI site is underlined) and 5′-TTTCTAGAACAGCCGCCTGATGTGACGT-3′ (an XbaI site is underlined). The obtained DNA fragment was cloned into PstI and XbaI sites of pKGLP2-BN159_4422-down/apra, resulting in pKGLP2-ΔBN159_4422. To construct a Δ*BN159_4422* strain, this plasmid was transferred into *S. davawensis* using a conjugation method described below. The desired recombination was confirmed by PCR using primer sets, 5′-GCCAGTCCTTCCACCGCGTC-3′ (primer 1 in Figure S2A) plus 5′-GGCGACAGCCCTGGGTCAAC-3′ (primer 2) and 5′-CCCATCTTCGAGGGGCCGGA-3′ (primer 3) plus 5′-CTCCTTCGGGGTGCCGTTCC-3′ (primer 4).

Construction of pTYM19ep-BN159_4422-4421

A DNA fragment harboring *BN159_4422* and *BN159_4421* was amplified by PCR using primers 5′-AACATATGACCACCAGACAGCACAC-3′ (an NdeI site is underlined, and the start codon of *BN159_4422* is italicized) and 5′-TTAAGCTTTCATGCCGCCGACCGGGTGCG-3′ (a HindIII site is underlined, and the stop codon of *BN159_4421* is italicized). The obtained DNA fragment was cloned into NdeI and HindIII sites of pTYM19ep [18], resulting in pTYM19ep-BN159_4422-4421, in which the BN159_4422-4421 operon is located under the control of *ermE** promoter.

Construction of the ΔBN159_5485 (*desD* homolog) strain

The BN159_5485 disruptant was constructed by substituting the core region of *BN159_5485* with *aac(3)IV* by homologous recombination. An upstream fragment (~2000 bp) of *BN159_5485* was amplified by PCR using primers 5′-GCGGCCGCGCGATGAGCAGTTGATACGGGTCGT-3′ and 5′-AACCCGATAGCGGGGCTGTA-3′. For the amplification of a downstream fragment (~2000 bp), primers 5′-GACGGATTCGGCGAGGCTCA-3′ and 5′-GATTACGAATTCGATGACGCCGTCGTGGCGGGCAC-3′ were used. The *aac(3)IV* gene was amplified by PCR using primers 5′-CCCCGCTATCGGGTTGAATAGGAACTTCGGAATAG-3′ and 5′-CTCGCCGAATCCGTCAGCAAAAGGGGATGATAAGT-3′ and an *acc(3)IV*-containing DNA fragment as a template. The amplified fragments were cloned together into an EcoRV site of pKGLP2 using In-fusion (Takara Bio Inc.), resulting in pKGLP2-ΔBN159_5485. To construct a Δ*BN159_5485* strain, this plasmid was transferred into *S. davawensis* using the conjugation method described below. The desired recombination was confirmed by PCR using a primer set, 5′-ATCGACCGCGCCAACCTCTA-3′ (primer 5 in Figure S2C) plus 5′-TCGGTGAAGTACTCGGCGAC-3′ (primer 6).

Conjugational transfer

E. coli strain ET12567/pUZ8002 harboring a plasmid for gene disruption was inoculated into 100 mL of Luria-Bertani (LB) medium and incubated at 37°C until OD_{600} reached 0.4–0.6. The cells were harvested and washed twice with LB medium and resuspended in 5 mL of LB medium. *S. davawensis* spores in 100 μL of 20% glycerol were suspended in 0.5 mL of TSB medium and incubated at 50°C for 10 min. The spores and *E. coli* cells were mixed and inoculated on MS agar plate containing 10 mM $MgCl_2$ and

60 mM $CaCl_2$. After incubation at 30 °C for 18 h, the plate was overlaid with an antibiotics solution (nalidixic acid [0.75 mg/mL] and apramycin [0.75 mg/mL] in 1 mL of sterile water). After incubation at 30 °C for 1 week, several apramycin-resistant *S. davawensis* colonies were obtained, in which the plasmid was integrated into the chromosome through single-crossover recombination. For the second recombination, the strains were repeatedly incubated on MS agar with apramycin (50 mg/L) for few days. Double crossover mutants were selected by blue-white selection using *gusA* on pKGLP2 and X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt). The spores harvested from MS agar were inoculated on Bennett maltose agar containing 50 mg/L apramycin and 0.3% X-gluc. White colonies were selected as candidates for a double crossover mutant. The desired recombination was confirmed by PCR using primers described above.

Analysis of metabolites of *S. davawensis* in combined culture

The wild-type and mutant *S. davawensis* strains were inoculated into TSB medium and incubated at 30 °C for 3 days. In parallel, *T. pulmonis* was inoculated into TSB medium and incubated at 30 °C for 2 days. Portions of *S. davawensis* pre-culture (3 mL) and *T. pulmonis* pre-culture (1 mL) were together inoculated into 100 mL of A-3M medium in a K-1 flask. After incubation at 30 °C for 2 days, 5 mL of culture broth was harvested and incubated with 0.1 g of Amberlite FPX66 resin (Organo, Tokyo, Japan) at room temperature for 2 h. The resin and cells were together harvested using centrifugation and washed with distilled water. Then the metabolites were extracted from the resin and cells with methanol. The methanol extract was evaporated to dryness and dissolved in 200 µL of dimethyl sulfoxide (DMSO) for LC-ESIMS analysis with a 1100 series spectrometer (Agilent Technologies, Santa Clara, CA) coupled to a High-Capacity Trap Plus system (Bruker Daltonics, Billerica, MA) equipped with a Cosmosil π NAP Packed column (2.0ID × 150 mm; Nacalai Tesque, Kyoto Japan). Compounds were eluted with a linear gradient of water and acetonitrile containing 0.1% formic acid. The flow rate was 0.4 mL/min. The gradient elution profile started with 2% acetonitrile and kept 2% for 2 min. The acetonitrile concentration was gradually increased to 50% in 28 min, to 100% in 2 min, and kept 100% for 2 min. The column was re-equilibrated with 2% acetonitrile for 3 min before the next injection. Absorbance at 210 nm was monitored.

Isolation and structural elucidation of compounds

For isolation of desferrioxamine derivatives, the wild-type *S. davawensis* strain was co-cultured with *T. pulmonis* in multiple flasks as described above. After the addition of Amberlite FPX66 resin (0.02 g/mL culture), the culture broth was incubated at room temperature for 2 h for the adsorption of compounds. The cells and FPX66 resin were harvested by filtration, and the compounds were extracted with methanol. The solvent was evaporated to dryness, and the residual materials were dissolved in methanol and subjected to medium-pressure liquid chromatography (MPLC; Purif-Compact A, Shoko Scientific, Tokyo, Japan) with a silica gel column (SIZE60; Shoko Scientific). Absorbed compounds were eluted with a linear gradient of chloroform/methanol from 100/0 to 0/100 for 20 min. The fraction containing the target compounds was evaporated to dryness. The residual materials were dissolved in methanol and subjected to high-performance liquid chromatography (HPLC; Shimadzu, Tokyo, Japan) equipped with Cosmosil π NAP column (10ID × 250 mm, Nakalai Tesque) and Cosmosil 5C18 AR-II column (10ID × 250 mm, Nakalai Tesque). In both cases, the target compounds were eluted in a linear gradient of water and methanol containing 0.1 % formic acid. As a result, compounds **1** (1 mg from 6 L of fermentation broth), **2** (11 mg from 9 L), and **3** (4.8 mg from 6 L) were isolated. The ^{1}H NMR, ^{13}C NMR, COSY, HMBC, HMQC, and TOCSY spectra were recorded in DMSO-*d*6 on JNM-A500 NMR System (JEOL, Tokyo, Japan).

Siderophore assay

Desferrioxamine derivatives (**1**, **2**, and **3**) were suspended in deionized water, and an equal volume of 5 mM $FeCl_3$ was added to the solution. Then, these samples were analyzed with LC-MS. The analytical conditions were the same as those in the analysis of *S. davawensis* metabolites.

Results and discussion

In silico analysis of a putative secondary metabolite biosynthesis gene cluster containing *creE* and *creD* homologs in *S. davawensis*

By BLAST search using CreE and CreD as queries, *creE* (*BN159_4422*) and *creD* (*BN159_4421*) homolog genes were discovered in the genome of *S. davawensis* JCM4913 [19] (Table 1 and Fig. 2). BN159_4422 and BN159_4421 showed 56.3% and 65.9% identity to CreE and CreD, respectively, of *S. cremeus*. The putative gene cluster containing *BN159_4422* and *BN159_4421* possesses several other genes that seem to be involved in secondary metabolite biosynthesis (Table 1 and Fig. 2). For example, *BN159_4424* encodes a TrpE(G)-like protein, which is involved in anthranilate biosynthesis. Interestingly, *S. davawensis* has another copy of *trpE* (*BN159_6401*) and BN159_6401 is highly homologous to TrpE proteins of other *Streptomyces* species

(e.g., SGR_5465 of *Streptomyces griseus*, 88.3% identity; SAV_6171 of *Streptomyces avermitilis*, 95.9%; SCO2043 of *Streptomyces coelicolor* A3(2), 92.7%). In contrast, BN159_4424 shows very poor homologies to these enzymes (around 10% identity). This fact suggests that BN159_6401 and BN159_4424 should be involved in tryptophan biosynthesis and secondary metabolism, respectively. Furthermore, this gene cluster encodes two putative ligases (BN159_4426 and BN159_4430) and two putative acyl carrier proteins (BN159_4427 and BN159_4431). The presence of these genes suggests that this gene cluster should be responsible for the biosynthesis of a secondary metabolite(s). However, it was very difficult to predict their chemical structures by bioinformatic analysis.

In vitro analysis of CreE and CreD homologs (BN159_4422 and BN159_4421) from *S. davawensis*

To confirm the enzymatic functions of the CreE and CreD homologs of *S. davawensis*, we produced C-terminally His-tagged BN159_4422 and N-terminally His-tagged BN159_4421 in *E. coli* and purified them by Ni^{2+} affinity chromatography (Figure S1A). The recombinant enzymes were used for the nitrous acid formation assay described previously. [16] As expected, these recombinant proteins produced nitrous acid from L-aspartic acid in the presence of NADPH and FAD (Figure S1B). This result clearly shows that CreE (BN159_4422) and CreD (BN159_4421) homologs from *S. davawensis* have the same functions as CreE and CreD from *S. cremeus*.

Natural products produced by the *creE* and *creD* homolog-containing gene cluster

To identify the natural products produced by the gene cluster containing the *creE* and *creD* homologs, we constructed a *BN159_4422* disruptant (Δ*BN159_4422*) by substituting the core region of *BN159_4422* with an apramycin resistance gene (Figure S2A and B) and compared its metabolic profile with that of the wild-type strain. Although we cultivated these two strains in various conditions, we could not observe any significant differences in their metabolic profiles (Fig. 3a, b). Thus, we assumed that this gene cluster is silent under usual laboratory conditions, and therefore we decided to use combined-culture to awaken the gene cluster. The two *S. davawensis* strains were individually co-cultured with *T. pulmonis* in A-3M medium. Comparison of their metabolic profiles showed that three compounds (**1**, **2**, and **3**) were detected only in the wild-type strain (Fig. 3d, e). These compounds were not produced when *S. davawensis* and *T. pulmonis* were cultured alone (Fig. 3a, c), indicating that the compounds were produced by *S. davawensis* and that its production was activated by co-cultivation with *T. pulmonis*. When the *BN159_4422-4421* operon was introduced into the Δ*BN159_4422* strain using the chromosome integrative vector pTYM19ep, the production of **1**, **2**, and **3** was restored (Figure S3). Taken together, these results clearly show that the *creE* and *creD* homologs are responsible for the biosynthesis of **1**, **2**, and **3**.

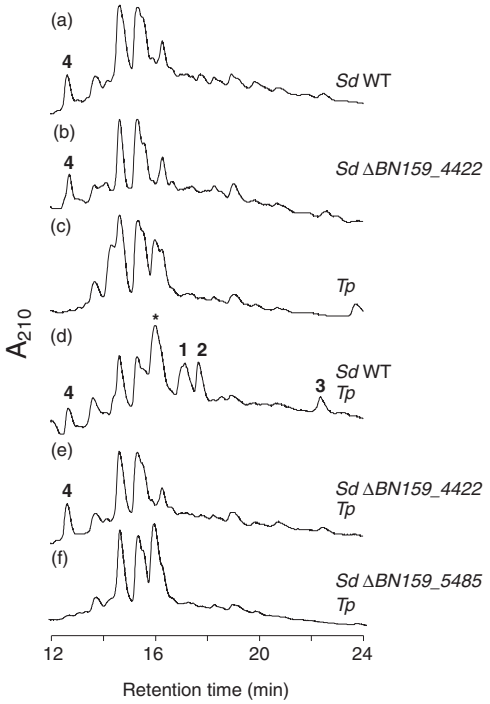

Fig. 3 LC-MS analysis of metabolites produced by the wild-type and mutant *S. davawensis* strains in pure culture and combined-culture with *T. pulmonis*. *BN159_4422* and *BN159_5485* encode CreE and DesD homologs, respectively. *Sd* and *Tp* indicate *S. davawensis* and *T. pulmonis*, respectively. The peak marked with an asterisk was concluded not to be related with the CreE and CreD homologs because it was observed occasionally

Isolation and structural elucidation of compounds 1, 2, and 3

The culture was scaled up for the purification of compounds **1**, **2**, and **3**. These three compounds were purified by silica gel chromatography using MPLC and reverse-phase chromatography using HPLC. High-resolution mass spectrometry (HR-MS) showed that the molecular formulae of sodium adducts of compounds **1**, **2**, and **3** were $C_{32}H_{54}N_6O_{11}Na$ (*m/z* 721.37482; calcd. for *m/z* 721.37428), $C_{33}H_{56}N_6O_{11}Na$ (*m/z* 735.39047; calcd. for *m/z* 735.38993), and $C_{33}H_{56}N_6O_9Na$ (*m/z* 703.40065; calcd. for *m/z* 703.40010), respectively. The compounds were further analyzed by 1D and 2D NMR (Table 2 and Fig. 4). Comparison of ^1H and ^{13}C NMR spectra with several desferrioxamine derivatives suggested that these compounds are desferrioxamine B derivatives with different modifications

Table 2 ^{1}H and ^{13}C NMR data for **1**, **2**, and **3**

Position	**1**		**2**		**3**	
	δ_C	δ_H *mult* (*J* in Hz)	δ_C	δ_H *mult* (*J* in Hz)	δ_C	δ_H *mult* (*J* in Hz)
1	20.9	1.92, s	20.9	1.91, s	20.9	1.92, s
2	170.6	—	170.6	—	170.7	—
3-NOH	—	—	—	—	—	—
4	47.5	3.41, t (6.5)	47.5	3.42, t (6.6)	47.3	3.41, t (7.0)
5	26.6	1.46, m	26.5	1.44, m	26.5	1.45, m
6	24.0	1.17, m	24.2	1.16, m	24.0	1.17, m
7	29.3	1.34, m	29.3	1.33, m	29.3	1.34, m
8	38.9	2.96, q (6.1)	38.9	2.95, q (6.1)	38.9	2.96, q (6.4)
9-NH	—	7.73, s	—	7.71, s	—	7.74, s
10	171.6	—	171.6	—	171.8	—
11	30.4	2.23, t (6.9)	30.4	2.22, t, (6.9)	30.4	2.22, t (7.1)
12	28.1	2.53, t (7.0)	28.1	2.53, t (7.0)	28.0	2.53, t (6.4)
13	172.6	—	172.6	—	172.5	—
14-NOH	—	—	—	—	—	—
15	47.5	3.41, t (6.5)	47.5	3.42, t (6.6)	47.3	3.41, t (7.0)
16	26.5	1.46, m	26.5	1.44, m	26.5	1.45, m
17	24.2	1.17, m	24.2	1.16, m	24.0	1.17, m
18	29.3	1.34, m	29.3	1.33, m	29.3	1.34, m
19	38.9	2.96, q (6.1)	38.9	2.95, q (6.1)	38.9	2.96, q (6.4)
20-NH	—	7.73, s	—	7.71, s	—	7.74, s
21	171.6	—	171.6	—	171.8	—
22	30.4	2.23, t, (6.9)	30.4	2.22, t, (6.9)	30.4	2.22, t (7.1)
23	28.1	2.53, t, (7.0)	28.1	2.53, t, (7.0)	28.0	2.53, t (6.4)
24	172.6	—	172.6	—	172.5	—
25-NOH	—	—	—	—	—	—
26	46.8	3.46, t, (6.5)	47.5	3.44, t, (6.5)	47.5	3.42, t (7.7)
27	24.0	1.49, m	29.0	1.64, m	26.3	1.49, m
28	26.6	1.60, m	23.3	1.22, m	23.6	1.18, m
29	44.7	3.39, t (7.8)	26.4	1.51, m	30.5	1.61, m
30	—	—	45.0	3.36, t (8.0)	46.8	3.77, t (7.2)
1′-N	—	—	—	—	—	—
2′	91.7	—	91.7	—	130.4	—
3′	196.0	—	196.1	—	106.9	6.11, s
4′	108.0	—	108.1	—	123.6	—
5′	167.0	8.73, s	166.9	8.74, s	126.7	7.42, s
6′	21.8	1.22, s	21.8	1.21, s	12.0	2.11, s
7′	193.6	—	193.1	—	195.4	—
8′	33.6	2.58, m	28.2	2.58, m	31.9	2.58, m
9′	8.9	0.92, t (7.2)	8.9	0.92, t (7.2)	9.5	0.97, t (7.4)

(Table 2) [20–22]. These structures were further supported by MS/MS analysis (Figure S4) [22]. The fragment ions observed were consistent with the cleavage at peptide bonds. Further analysis of unassigned NMR signals clearly showed that these compounds are novel desferrioxamine derivatives with either of two unusual N-containing five-membered ring structures (Fig. 4). The terminal amine of desferrioxamine B (and its analog) is apparently modified to produce these heterocyclic structures (a pyrrole ring in compound **3** and more oxidized one in compounds **1** and **2**). In the structures of **1** and **2**, the terminal heterocyclic structure is common and the difference is originated from the desferrioxamine chain length; **1** has a one-carbon shorter chain than **2**. Meanwhile, in the structures of **2** and **3**, the desferrioxamine chain length is common, but their terminal heterocyclic structures are different. According to their structures, we named these compounds desferrioxamine I_{2a} (**1**), I_{1a} (**2**), and I_{1b} (**3**).

Analysis of the putative desferrioxamine biosynthetic gene cluster

Although involvement of the *creE* homolog in the biosynthesis of these desferrioxamine derivatives was obvious, no genes related to desferrioxamine biosynthesis are encoded by the gene cluster containing the *creE* and *creD* homologs. Instead, a putative desferrioxamine biosynthetic gene cluster is present at a different locus in the genome (BN159_5485-5490, Figure S5 and Table S1) [23, 24]. To examine whether this biosynthetic gene cluster is related to the biosynthesis of **1**, **2**, and **3**, a *BN159_5485* (*desD* homolog) disruptant (Δ*BN159_5485*) was constructed (Figure S2C and D). As expected, the Δ*BN159_5485* strain did not produce **1**, **2**, or **3** in the combined-culture with *T. pulmonis* (Fig. 3f). Interestingly, production of another compound (**4**) was also abolished in the disruptant (Fig. 3f). By MS/MS analysis, this compound was identified to be desferrioxamine B (**4**) (Figure S4) [25]. Taken together, we concluded that the *BN159_5485-5490* operon is responsible for the biosynthesis of desferrioxamine derivatives including compounds **1**, **2**, **3**, and **4** in *S. davawensis*. We speculate that desferrioxamine B (**4**) should be a biosynthetic

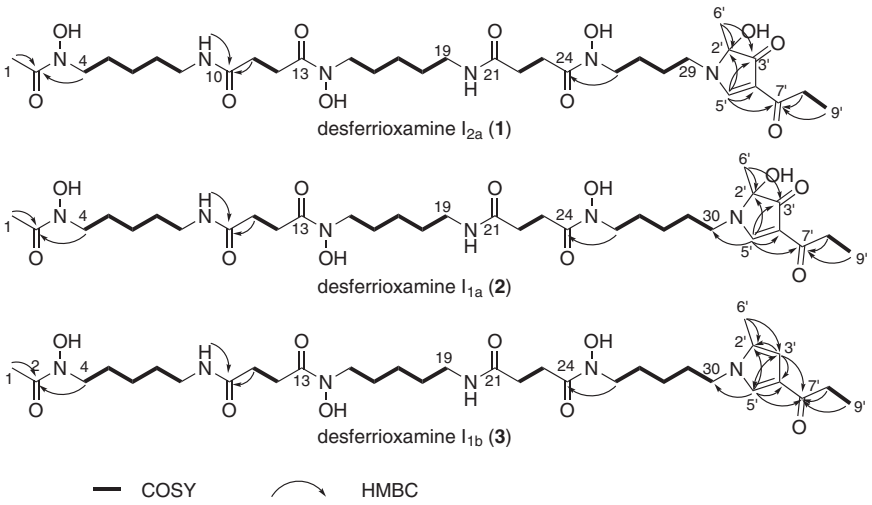

Fig. 4 Structures of **1**, **2**, and **3**. Key correlations of ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMBC are depicted as illustrated in the lower part

intermediate of **2** and **3**. We also speculate that a desferrioxamine B analog (**5**), which has a shorter carbon chain by one methylene than desferrioxamine B (**4**) (Fig. 1), should be a biosynthetic intermediate of **1**; a trace amount of **5** was also detected in the culture broth of the wild-type *S. davawensis* strain (both pure culture and combined-culture with *T. pulmonis*) (Figure S4). It should be noted that production of **4** and **5** was also observed in the pure culture of both the *S. davawensis* wild-type and Δ*BN159_4422* strains. This result indicates that the *BN159_5485-5490* operon responsible for the biosynthesis of **4** and **5** is expressed under the normal culture condition and that the gene cluster containing the *creE* and *creD* homologs is awakened by combined culture, which results in the conversion of **4** and **5** into the novel desferrioxamine derivatives **1**, **2**, and **3**.

Siderophore assay of 1, 2, and 3

According to their structures, **1**, **2**, and **3** were expected to have siderophore activity. To confirm this, these compounds were incubated with Fe^{3+} ion and analyzed by LC-MS (Figure S6). As a result, these compounds gained 53 Da, which corresponds to one Fe^{3+} ion. In addition, a significant change in the UV spectra was observed: new λ_{max} appeared around 430 nm. These results clearly show that compounds **1**, **2**, and **3** act as a siderophore.

Conclusion

In this study, we discovered three novel desferrioxamine siderophores produced by *S. davawensis* under combined-culture with *T. pulmonis* through genome mining of the ANS pathway. The compounds are probably biosynthesized by modifying desferrioxamine B (**4**) or its analog (**5**), which are produced by the function of the *BN159_5485-5490* operon (Fig. 1b). However, because the terminal heterocyclic structures in **1**, **2**, and **3** have not been reported so far, it is difficult to predict how these structures are biosynthesized. Because BN159_4422 and BN159_4421 were shown to produce nitrous acid in vitro and these genes are required for the biosynthesis of **1**, **2**, and **3** in vivo, nitrous acid probably plays an important role in the biosynthesis of these terminal heterocyclic structures. It is our future challenge to reveal the biosynthetic pathway for these novel desferrioxamine derivatives including the role of nitrous acid in this pathway. However, involvement of the ANS pathway in the biosynthesis of the unique terminal heterocyclic structures of these novel desferrioxamine derivatives provided an important example for the diverse usage of the ANS pathway for the biosynthesis of various secondary metabolites in actinomycetes. This study also indicates that the ANS pathway could be a useful source of information for the discovery of novel natural products as well as novel secondary metabolite gene clusters.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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