Heterologous Production of Desferrioxamines with a Fusion Biosynthetic Gene Cluster

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Desferrioxamines E (1), D2 (2), X1 (3), and X2 (4), four macrocyclic N-hydroxy-N-succinyl diamine-based siderophores, were produced efficiently by heterologous expression of a fusion biosynthetic gene cluster. This expression system consisted of three genes (mbsA-C) from marine metagenomic DNA and one gene (dfoC) from the terrestrial bacterium Erwinia amylovora. The first three genes are functional in the production of the common monomers N-hydroxy-N-succinyl cadaverine (5, HSC) and N-hydroxy-N-succinyl putrescine (6, HSP), whereas dfoC catalyzes the oligomerization and the macrocyclization reactions of compounds 5 and 6 to form compounds 1–4. This fusion gene cluster system provides a convenient expression platform for various biosynthetic genes of HSC-HSP based siderophores by simply switching the fourth gene by the cassette process.

Key words: siderophore; desferrioxamines; bisucaberins; heterologous production; macrocycle

Siderophores are bacterial metabolites that possess an affinity for ferric ion, an essential element for almost all organisms, and they are functional in iron uptake and transport. Bioavailable ferric iron is limited in the biosphere and is one of the elements that limit bioproduction. Therefore, a wide variety of bacteria produces siderophores and utilize them for ferric iron incorporation. Siderophores also attract the interest of chemical ecologists, because some bacteria depend on exogenous siderophores produced by neighboring strains for their own growth, and it has been hypothesized that complex siderophore chemical communication networks exist among environmental bacteria. It reported that siderophores produced by pathogenic bacteria that attack plants and fish enhance the bacterium’s virulence. Siderophores also have applications in human medicine. A derivative of the siderophore desferrioxamine is used as a clinical drug to treat iron poisoning, and also reportedly inhibits Mycobacterium sp. biofilm formation.

Recently, we obtained a novel biosynthetic gene cluster for bisucaberin (7), a macrocyclic dimer of compound 5, from a marine metagenomic library, and achieved heterologous production of 7 with Escherichia coli as host organism. To date, several HSC-based siderophores, including desferrioxamine E (1), bisucaberin (7), and bisucaberin B (8), and some of their biosynthetic genes, have been reported from various bacteria. The general biosynthetic pathway and composition of the biosynthetic gene cluster of HSC-based siderophores are shown in Fig. 1. The first three enzymes (A–C) are functional in the formation of HSC, a common monomer for this type of siderophore, by catalyzing a sequential reaction leading from lysine to HSC (5). Following HSC formation, the fourth enzyme (D) catalyzes both the oligomerization and the macrocyclization reaction to produce the final products. This biosynthetic scheme suggests that the production of structurally diverse HSC-based siderophores is possible simply by switching the last gene (encoding enzyme D) with either a homolog available in a DNA database or with a mutant gene. Enzyme Ds reported for various HSC-based siderophore producers are nearly the same size (about 600 residues) and show 40–60% amino acid sequence identity. They also share the functions of oligomerizing 2–4 monomers and macrocyclizing them, but the portion defining the final products remains unidentified. Engineering a diverse array of HSC-based siderophore biosynthetic clones might provide insight into the largely unknown
molecular mechanisms underlying regulation of the oligomerization and the macrocyclization reactions.

In this study, we constructed a combined HSC supply and a simple gene
D
-switching system (Fig. 3) as a platform to investigate not only the function of enzyme D homologs but also the production of structurally diverse analogs. Here we report the preparation of an in vivo HSC supply clone with the genes mbsA-C from our marine metagenome collection. We also describe the construction of a fusion gene cluster with the dfoC gene from the terrestrial plant pathogenic bacterium Erwinia amylovora as a putative enzyme D homolog, which resulted in the efficient production of desferrioxamines E (1), D2 (2), X1 (3), and X2 (4).

Results and Discussion

Construction of the HSC supply clone

A 3,839-bp DNA fragment including the putative promoter region and the first three genes (mbsA-C) was PCR-amplified from a bisucaberin (7)-producing clone obtained from a deep-sea metagenome. In the original cluster, the stop codon of mbsC overlapped with the start codon of mbsD by 1 bp (that is, there was no margin between the genes). In the HSC supply clone that we constructed, however, a SalI restriction enzyme recognition site was added downstream of mbsC for easy insertion of various gene Ds (Fig. 3). A set of the three mbs genes amplified was subcloned into the phargemid vector, and then transformed into E. coli to form a common HSC monomer (5) supply clone.

Analysis of the culture medium of the constructed clone by LC-MS revealed the presence of a compound with an m/z of 219, corresponding to HSC, and no such ion was observed in the negative control (Fig. 4). Unexpectedly, a molecular ion at m/z 205 that corresponded to HSP (6) was also observed (Fig. 4). These compounds were purified by column chromatography and isolated by reversed phase HPLC (yields from a 200-mL culture; 5, 4.6 mg; 6, 6.5 mg). The compounds were identified by NMR and LC-MSMS (see Fig. 4 and Supplemental Information; see Biosci. Biotechnol. Biochem. web site). These results suggest that enzymes MbsA, B, and C efficiently catalyze reactions involving not only lysine and its metabolites but also ornithine-derived metabolites. Other condensed molecules (e.g., compounds 1–4, 7, and 8) were not detected by LC-MS analysis. These results revealed that the constructed clone can produce both HSC and HSP efficiently, but lacks the capacity for further oligomerization and macrocyclization necessary to produce the bacterial siderophores.

Construction of the fusion gene cluster clone

As the first target of the fusion gene cluster system, we employed putative desferrioxamine E biosynthetic genes dfoA-C from the plant pathogen Erwinia amylovora of the phylum Proteobacteria. This species reportedly produces desferrioxamine E (1) and several minor analogs and the production of these siderophores is related to enhancement of the bacterium’s pathogenicity. Although a candidate biosynthetic gene cluster has been reported in the genomic data for this
strain on the basis of sequence homology, the function of the gene cluster has yet to be confirmed experimentally.15)

The putative desferrioxamine biosynthetic gene cluster of
\( E. \) amylovora consists of three ORFs (dfoA-C). According to a BLAST search, the N-terminal 189 residues of the third enzyme, DfoC (784 aa), showed high sequence homology to that of enzyme Cs identified in the other organisms. On the other hand, the C-terminal part consisted of 595 residues corresponds to enzymes D in related clusters, which suggests that it is a multifunctional hybrid enzyme (Fig. 5). The 190th methionine residue of DfoC might be the start codon of an ancient separated gene \( D \), and is expected to be usable in translation initiation of the recombinant gene.

Fig. 4. Analysis of the Monomer Supply Clone.
Selected ion monitoring (SIM) LC-MS chromatogram of the culture medium (black, monomer supply clone; blue, vector only) at \( m/z \) 219 and 205 (top). LC-MSMS analysis of compound 5 using \( m/z \) 219 as the precursor ion (middle). LC-MSMS analysis of compound 6 using \( m/z \) 205 as the precursor ion (bottom).

Fig. 5. Graphical Scheme Illustrating the Construction of the mbsA-C and dfoCC Fusion Gene Cluster.

A 1,797-bp DNA fragment encoding 595 amino acids of the C-terminal portion of DfoC (DfoC̄), corresponding to enzyme D, was chemically synthesized. Recognition sequences for the restriction enzymes Sall and Apal were added at the 5’ and 3’ ends respectively of the synthetic DNA fragment. The synthetic \( dfoC \) gene was inserted directly downstream of \( mbsC \) by means of the Sall and Apal recognition sites, and then transformed into \( E. \) coli competent cells by electroporation to produce the fusion gene-cluster clone (Fig. 5).

Isolation and identification of products

The clone containing the fusion gene cluster exhibited potent siderophore activity as confirmed in a CAS assay, indicating that the recombinant truncated \( E. \) amylovora DfoC expressed in the heterologous host was functional (Fig. 6). To identify the products, the clone was cultured in antibiotic-selection medium for 4 d, and then the medium was recovered and fractionated by repetitive column chromatography guided by CAS assay results. Final purification by HPLC yielded four active compounds, desferrioxamines E (1), D2 (2), X1 (3), and X2 (4), isolation yield, 2.7 mg (1) and 5.3 mg (2) for 100 mL of culture, and 7.1 mg (3) and 1.2 mg (4) for 1.0 L of culture.

The structures of compounds 1 and 2 were determined unambiguously by HR-ESIMS and 1D- and 2D-NMR analysis including COSY, ROESY, HMQC, and HMBC spectra (Supplemental Information). Identification of the minor analogs, 3 and 4, was done based on the HR-ESIMS data as well as a comparison of the 1D-NMR data with those of 1 and 2. Compounds 1–4 exhibited similar \(^1\)H NMR spectra, except for the signal intensity of H-7 (about \( \delta _{1H} \) 1.20), two-thirds of the other

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methylenes in the spectrum of compound 2, and one-third in the case of 3. Furthermore, this signal was not observed in the $^1$H NMR spectrum of 4. The molecular formulae of the compounds (1, C$_{27}$H$_{48}$N$_{6}$O$_{5}$; 2, C$_{36}$H$_{60}$N$_{10}$O$_{8}$; 3, C$_{25}$H$_{42}$N$_{8}$O$_{6}$; 4, C$_{25}$H$_{42}$N$_{8}$O$_{6}$), deduced from the HR-ESIMS data, indicated that compound 1 is a macrocyclic trimer of HSCs (5), while one to three HSCs were replaced with HSPs (6) in compounds 2–4 respectively. Thus we confirmed that the fusion gene cluster clone constructed efficiently produces the predicted macrocyclic trimer desferrioxamines E (1), D2 (2), X1 (3), and X2 (4). We also found that the putative E. amylovora biosynthetic gene cluster is functional in the production of compounds 1–4. Moreover, only the C-terminal portion of DfoC (DfoC$^C$) can catalyze both the trimerization and the macrocyclization of HSC (5) and HSP (6).

The production of compounds 1–4 (27, 53, 7.1, and 1.2 mg/L respectively) by the fusion gene cluster clone constructed was much higher than that of bisucaberin (7, 8.4 mg/L) produced by an original clone containing mbsA-D, suggesting that heterologous expression of the truncated dfoC gene proceeds efficiently in E. coli. Recombinant enzyme DfoC$^C$ recognized both HSC (5) and HSP (6) as substrate, preferring 5 to 6 at ratio of about 5:1, judging from the amount of 1–4 isolated. In the LC-MS analysis, all four predicted trimers were observed, while the tetramers reported for the original strain were not detected for the fusion gene cluster clone (Supplemental Information). This result suggests that the possibility of influence of the truncated N-terminal part of DfoC on the regulation of the oligomerization reactions.

Our new siderophore production platform makes possible easy gene switching with efficient expression, enabling rapid analysis of large numbers of condensation enzymes. Recently we found a marine bacterium that produces bisucaberin B (8), which is a linear dimer of HSC, that does not produce cyclic counterpart 7. We expect that a comparison of mutant or hybrid enzymes as between the producers of 7 and 8 by means of our production platform would provide additional insight into the molecular mechanisms underlying the oligomerization and the macrocyclization reactions, which might be shared with other HSC/HSP-based siderophore biosynthetic processes. Additionally, the equally efficient production of monomers 5 and 6 by MbsA-C suggests that these enzymes have relatively wide substrate recognition capacities. Thus the chemoenzymatic production of structurally diverse monomers might be possible simply by adding a variety of diamine precursors. Modification of enzymes might also result in the production of novel final products. Studies using the present production platform are in progress and are to be reported elsewhere.

Conclusions

By cloning genes mbsA-C of the marine metagenome-derived bisucaberin biosynthetic pathway, a platform for the efficient production of common monomers HSC (5) and HSP (6) was constructed. The putative desferrioxamine E (1) biosynthesis gene dfoC$^C$ of E. amylovora was easily incorporated into this system to form a fusion gene cluster clone. Heterologous expression of these genes resulted in high-yield production of desferrioxamine E (1) and of HSP-containing analogs desferrioxamines D2 (2), X1 (3), and X2 (4). Our results experimentally indicate that the putative E. amylovora genes are involved in desferrioxamine production, and that only the C-terminal portion of DfoC is necessary for efficient catalysis of the oligomerization and the macrocyclization reactions. The system we constructed is a convenient platform for investigating the functions of various oligomerization and macrocyclization enzymes. Easy gene switching makes possible the production of structurally diverse analogs.

Experimental

General experimental procedures. Low- and high-resolution ESI mass spectra were collected on an Exactive mass spectrometer (Thermo Scientific, Waltham, MA). NMR spectra were recorded on an ECP-400 NMR spectrometer (JEOL, Tokyo) at 400 MHz for $^1$H and at 100 MHz for $^{13}$C. $^1$H and $^{13}$C chemical shifts in NMR spectra were referenced to the solvent peaks: $^1$H 2.49 and $^{13}$C 39.5 for DMSO-d$_6$. UV absorption in the CAS assay was measured on a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). Preparative and analytical HPLC analyses were done with a Prominence HPLC system equipped with a photodiode array detector (Shimadzu, Kyoto, Japan). LC-MS and LC-MSMS analyses were done with an LCMS-8040 LC-MSMS system equipped with a Prominence HPLC system (Shimadzu). DNA sequences were determined with a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and a 3130xl Genetic Analyzer (Applied Biosystems). For DNA cloning, NEB 10-beta–competent E. coli cells (New England BioLabs, Ipswich, MA), which did not show any siderophore production activity.
in the CAS assays, were used. Electroporation was done with a MicroPulser electroporator (Bio-Rad, Hercules, CA). A GeneAtlas thermal cycler (Astec, Fukuoka, Japan) and a KOD Plus Neo PCR kit (Toyobo, Osaka, Japan) were used to amplify DNA fragments. Oligo DNAs for PCR and DNA sequencing were purchased from Hokkaido System Science (Sapporo, Japan). Unless otherwise indicated, all chemicals were purchased from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries (Osaka, Japan).

Artificial gene construction. A 1,797-bp DNA fragment encoding 595 amino acids of the C-terminal portion of Erwinia amylovora ATCC BAA-2158 DfoC (DfoCζ, accession no. FR719197) was synthesized chemically by Bioneer (Daejeon, Korea). Recognition sites for restriction enzymes Sall and ApaI were added at the 5’ and the 3’ ends respectively (for sequence, see Supplemental Information).

Construction of monomer supply clone. A DNA fragment corresponding to the mbhA-C was amplified by PCR with bisucaberin chemically synthesized strain (Aptc) as template DNA with the following primer set: forward: 5-ACG TCATTGTGTGTC-3, reverse: 5-TTT GCTGCTCTGCTTG-3 (underlined sequences show the Sall and ApaI recognition sites respectively). PCR was done following the protocol provided by the manufacturer, initial denaturation at 95°C for 2 min, followed by 30 cycles of 2°C for 30 s, 60°C for 30 s, and 72°C for 150 s. The 3,847-bp PCR amplicon was purified by agarose gel electrophoresis and extracted from the gel with an AxyPrep DNA Gel Extraction Kit (Axygen, Union City, CA). The purified PCR product was digested with BstEII and SalI (Takara Bio, Otsu, Japan), and then ligated into the pBSK+ phagemid vector (Agilent Technologies, Santa Clara, CA) with T4 DNA ligase (Takara Bio) following the manufacturers’ protocols to construct the monomer-production plasmid. The plasmid was then transformed into NEB 10-beta-competent E. coli cells by electroporation. The transformation mixture was spread onto LB agar (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl and 10 g of agar in 1 L of deionized water) plates containing 30 μg/mL of chloramphenicol for cultivation of the monomer-producing clone.

LC-MS and LC-MSMS analysis of monomers. The monomer-producing and negative control (vector only) clones were cultured at 30°C for 4 d with shaking at 200 rpm in LB medium containing 30 μg/mL of chloramphenicol and 0.1 mM IPTG, and then incubated at 30°C for 4 d with shaking at 200 rpm. After centrifugation, the active molecules in the supernatant were adsorbed onto C18 resin and eluted with MeOH. The CAS-active extract was fractionated by C18 resin open-column chromatography with stepwise gradient elution from pure water with aqueous MeOH (10%, 30%, 50%, 70%, and 100% MeOH). The active fractions eluted between 30% and 70% aqueous MeOH were combined and further purified by Sephadex G-10 column chromatography with water. Activity was found in the fractions eluting between 150 and 350 μL. The active fractions were combined and further fractionated by Sephadex LH20 gel filtration with 50% aqueous MeOH as eluent. The active fractions obtained were concentrated, and then a portion of the fraction (100 μL culture equivalent for 1 and 2, and 1.0 μL culture equivalent for 3 and 4) was purified by reversed phase semi-preparative HPLC (Inertsil ODS-3, 4.6 × 250 mm, GL Sciences, Tokyo). The active fractions were then further purified by Sephadex G-10 column chromatography with 10% aqueous MeOH as eluent. The active fractions obtained were concentrated, and then a portion of the fraction (100 μL culture equivalent for 1 and 2, and 1.0 μL culture equivalent for 3 and 4) was purified by reversed phase semi-preparative HPLC (Inertsil ODS-3, 4.6 × 250 mm, GL Sciences, Tokyo).

Monomer production and identification. The monomer-producing clone was precultured overnight in LB medium containing chloramphenicol and then inoculated into four 1-L flasks containing 400 mL of LB medium supplemented with 30 μg/mL of chloramphenicol and 0.1 mM IPTG, and then incubated at 30°C for 4 d with shaking at 200 rpm. After centrifugation, the active molecules in the supernatant were adsorbed onto C18 resin and eluted with MeOH. The CAS-active extract was fractionated by C18 resin open-column chromatography with stepwise gradient elution from pure water with aqueous MeOH (10%, 30%, 50%, 70%, and 100% MeOH). The active fractions eluted between 30% and 70% aqueous MeOH were combined and further purified by Sephadex G-10 column chromatography with water.

Production and identification of desferrioxamines. The fusion gene cluster clone was precultured overnight in LB medium containing chloramphenicol and then inoculated into four 1-L flasks containing 400 mL of LB medium supplemented with 30 μg/mL of chloramphenicol and 0.1 mM IPTG, and then incubated at 30°C for 4 d with shaking at 200 rpm. After centrifugation, the active molecules in the supernatant were adsorbed onto C18 resin and eluted with MeOH. The CAS-active extract was fractionated by C18 resin open-column chromatography with stepwise gradient elution from pure water with aqueous MeOH (10%, 30%, 50%, 70%, and 100% MeOH). The active fractions eluted between 30% and 70% aqueous MeOH were combined and further purified by Sephadex G-10 column chromatography with water. Activity was found in the fractions eluting between 150 and 350 μL. The active fractions were combined and further fractionated by Sephadex LH20 gel filtration with 50% aqueous MeOH as eluent. The active fractions obtained were concentrated, and then a portion of the fraction (100 μL culture equivalent for 1 and 2, and 1.0 μL culture equivalent for 3 and 4) was purified by reversed phase semi-preparative HPLC (Inertsil ODS-3, 4.6 × 250 mm, GL Sciences, Tokyo).

CAS agar plate assay. Chrome azur S (CAS) assays were performed basically according to a reported method. Ferric ion chelating CAS shows blue color (absorption maximum 630 nm), but once it loses ferric ion due to the presence of the other siderophores, the color turns to yellow, and the iron-free CAS has essentially no
absorption at 630 nm. CAS assay plates were prepared by mixing a stock solution of sterilized CAS (0.1 mM FeCl$_3$, 1.0 mM HCl, 1.0 mM CAS, and 2.0 mM cethyltrimetylammonium bromide) with melted LB agar (1:9). Paper discs (6 mm in diameter) containing absorbed test samples or test clones were placed on the CAS assay plates and kept overnight at room temperature. Then the diameters of the yellow halos around the test samples and colonies were measured.

**CAS solution assay.** Culture media or fractions were mixed with equal volumes of CAS assay solution (0.6 mM cetyltrimethylammonium bromide, 15 mM FeCl$_3$, 150 mM CAS, 0.5 M anhydrous piperazine, 0.75 M HCl) and kept at room temperature for 4 h, and then the absorption at 630 nm was measured.

**LC-MS analysis of the fusion gene cluster clone.** The fusion gene cluster clone was cultured at 30°C for 4 d with shaking at 200 rpm in LB medium containing 30 μg/mL of chloramphenicol and 0.1 mM IPTG. The resulting culture medium was passed through C18 resin to remove the inorganic salts and polar compounds. The MeOH eluted extract was dried and re-dissolved in 50% aqueous MeOH. A portion of the solution was analyzed by LC-MS (column, Inertsil ODS-3, 2 × 100 mm, GL Sciences; solvent, aqueous MeOH linear gradient from 0 to 80% over 15 min; flow rate, 0.2 mL/min; detection, scan from $m/z$ 550 to 820.

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