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Screening of Streptomycetes for Production of Desferrioxamines

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Desferrioxamine B is a hydroxamate-type siderophore originally isolated from *S. pilosus*, and has been used to treat thalassemia major patients by chelating overloaded iron. Multiple reports of production of desferrioxamines in streptomycetes were reported, however the distribution of desferrioxamines were still not clear. To examine the distribution of desferrioxamines in streptomycetes, we performed HPLC screening using 41 streptomyces strains. The production of desferrioxamines was detected in high percentage (78%, 32/41) of streptomycetes, which indicated that desferrioxamines were widely distributed in streptomycetes.

Key words: Siderophore, *Streptomyces,* Desferrioxamines, HPLC, ESI-MS.

Siderophores are low-molecular compounds that are produced by living organisms to sequester Fe^{3+} by chelating. Their biosynthesis is strictly regulated and triggered by growth condition with an insufficient iron level[1]. Many species of bacteria, fungi, algae and higher plants have been reported to produce siderophores in response to iron limitation[2-6]. In general, siderophores can be classified structurally as catecholes, phenolates, hydroxamates, and carboxylates. The most important functional discrepancies among them are variations in stability and in affinity for Fe^{3+}. After a siderophore chelates iron, the structure specific receptor to the siderophore is located in cell membrane and ferric siderophore complex is taken up into bacterial cell. The iron uptake systems of pathogenic bacteria using siderophores have been well studied for the sake of establishing efficient clinical treatments because iron acquisition was critical process for pathogen to invade and survive in host cells. In fact, the secretion of some siderophores was reported to enhance the virulence of the photogenic bacteria[7]. Due to these biological significances, many siderophores including pyochelin[8], yersiniabactin[9], petrobactin[10], and mycobactin[11] were isolated from pathogenic bacteria.

Among siderophores, desferrioxamine B (Fig.1) was a hydroxamate siderophore originally isolated from *S. pilosus*, and has been used to treat thalassemia major patients by chelating overloaded iron. Since desferrioxamines (Fig. 1) are produced by several species of *Nocardia*[12], *Streptomyces*[13-15], *Micromonospora*[16], *Arthrobacter*[17], *Chromobacterium*[17], and *Pseudomonas*[18], desferrioxamines seem to be distributed in broad range of bacteria. Recently, desferrioxamine E was reported to have the important physiological functions such as activation of the antibiotic

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production and aerial hyphae formation in *Streptomyces tanashiensis*. *Streptomycetes* are soil-dwelling bacteria constantly exposed to dryness and completion. Considering that the physiological activities such as antibiotic production and aerial hyphae formation are critical strategy for surviving, this result suggested that desferrioxamine E may be a signal compound across streptomycetes. However, so far it is not clear that the distribution of this important siderophores in streptomycetes. Clarification of production profile of desferrioxamines in streptomycetes will give the useful information regarding the distribution and biosynthesis of desferrioxamines. In this report, we performed HPLC quantification of desferrioxamines production using 41 strains of streptomycetes.

RESULTS AND DISCUSSION

The strains of streptomycetes were obtained from culture collection (NBRC, strain numbers were shown in Table 1), and all the strains except for NBRC3187 were type strains. Then each strain was cultured using 10 ml of iron deficient medium for 5 days. After centrifugation, the supernatant of culture broth (1 mL) was transferred into microtube. Then 30 µL of $FeCl_3$ was added to the supernatant to convert desferrioxamines into ferrioxamines. The immediate change of color to light red was observed. The 100 µl of each sample was subjected to HPLC analysis with UV/VIS detector set at 425 nm. As a result, two major peaks were observed on HPLC chromatogram at the retention time of 19.0 and 20.4 min as shown in Fig. 2. Identification was performed using ESI-MS spectrum. The compounds 1 (19.0 min) and 2 (20.4 min) gave the ion peaks at *m/z* $[M+H]^+$ 612.4 and 654.2. The retention time of compound 1 was identical with that of authentic ferrioxamine B (Sigma-Aldrich). Judging by the HPLC retention time and molecular weight, the compounds 1 and 2 were identified to be ferrioxamine B and E, respectively. The quantification was accomplished by peak area compared with that of authentic ferrioxamine B.

The production of desferrioxiamine/s was observed in 32 strains out of 41 strains as shown in Table 1. Then all strains fell into 4 groups by the production profiles of desferrioxamines; Group A produces desferrioxamines B and E; Group B produces only desferrioxamine B; Group C produces only desferrioxamine E; Group D produces none. The group A contained 15 strains including industrially important strain, *S. avermitilis*, which produced an antibiotic avermectin. The group B consisted of 8 strains which included *S. minoensis*, producer of an important antibiotic minocycline. The group C had 9 strains which included tetracycline producers, *S. rimosus*, *S. platensis,* and *S. aureofaciens*. The group D contained 9 strains which produced no siderophores in this experimental condition. The highest production of desferrioxamine B was observed in *S. odorifer* with the production of 10.5 mg/L. On the other hand, *S. noursei* was the highest producer of desferrioxamine E, and the production was as high as 27.0 mg/L.

Previous report indicated that desferrioxamine E had the important physiological function including activation of the antibiotic production and aerial hyphae formation in *S. tanashiensis*. Since aerial hyphae formation and antibiotic production are for self-defence, siderophore might function not only as a chelating substance but also as a signal compound to survive in competitive condition. In this paper, desferrioxamines were detected in high percentage (78%, 32/41) of streptomycete strains, which indicated that desferrioxamines were widely distributed in streptomycetes.

MATERIALS AND METHODS

General Methods

The chromatography system consisted of 2 HPLC pumps (Jasco, PU-980), a UV/VIS detector (Jasco, Model UV-970) and digital integration software (MacIntegrator II). ESI-TOF MS spectra were recorded by a JOEL JMS-T100LP mass spectrometer.

Bacterial strain

All strains shown in Table 1 were obtained from Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Japan.

Culture media

The iron deficient medium consisted of 2 g of K_2SO_4, 3 g of K_2HPO_4, 1 g of NaCl, 5 g of NH_4Cl in 1 L of deionized water. To remove ferric ions, the solution was stirred with 50 g of chelex-100 with

Na form (Bio-Rad) for 16 h. The solution was filtrated with paper filter (Whatman No.1) and added with the stock solutions: 100 µL of thiamine (20 mg/mL), 100 µL of $ZnSO_4 \cdot 7H_2O$ (20 mg/mL), 20 µL of $CuSO_4 \cdot 7H_2O$ (0.5 mg/mL), 20 µL of $MnSO_4 \cdot 4H_2O$ (3.5 mg/mL), followed by autoclaving. The separately sterilized solutions (10 mL each) of $CaCl_2 \cdot H_2O$ (10 mg/mL), glucose (250 mg/mL), and 0.5% yeast extract (Difco) were added to the medium.

Table 1. Production of desferrioxamines B and E in streptomycetes

Group	NBRC No.	Strain	Production (mg/L)	
			Desferrioxamine B	Desferrioxamine E
A	13450	*S. mirabilis*	4.0	1.4
	15394	*S. chrysomallus*	2.3	1.6
	14893	*S. avermitilis*	4.0	2.3
	12752	*S. canus*	3.9	2.1
	12864	*S. galbus*	5.6	3.8
	3517	*S. sayamaensis*	2.8	0.5
	15896	*S. aureorectus*	3.3	7.4
	13365	*S. odorifer*	10.5	2.9
	3561	*S. olivochromogenes*	7.2	8.2
	13814	*S. diastatochromogenes*	0.6	0.2
	13024	*S. capuensis*	0.9	2.4
	12803	*S. nitrosporeus*	0.7	1.7
	13370	*S. thermophilus*	1.9	9.0
	13455	*S. graminofaciens*	6.3	6.1
	100999	*S. mutomycini*	1.1	1.1
B	15797	*S. minoensis*	1.9	ND
	14062	*S. wedmorensis*	3.5	ND
	13398	*S. cirratus*	6.6	ND
	13438	*S. anandii*	8.2	ND
	12827	*S. virginiae*	6.0	ND
	3117	*S. antibioticus*	1.2	ND
	12919	*S. tanashiensis*	0.5	ND
	100760	*S. scabrisporus*	3.0	ND
C	12907	*S. platensis*	ND	8.5
	13054	*S. lincolnensis*	ND	14.8
	12907	*S. rimosus*	ND	14.5
	13058	*S. lydicus*	ND	24.7
	15452	*S. noursei*	ND	27.0
	12839	*S. antimycoticus*	ND	0.4
	12741	*S. atroolivaceus*	ND	2.4
	12833	*S. albofaciens*	ND	7.6
	12843	*S. aureofaciens*	ND	0.1
D	12797	*S. michiganensis*	ND	ND
	12852	*S. cinnamoneus*	ND	ND
	3187	*S. aureofaciens*	ND	ND
	15423	*S. levis*	ND	ND
	13441	*S. durhamensis*	ND	ND
	12784	*S. hawaiiensis*	ND	ND
	13373	*S. viridis*	ND	ND
	13868	*S. hygroscopicus*	ND	ND
	13348	*S. zaomyceticus*	ND	ND

ND - Not Detected

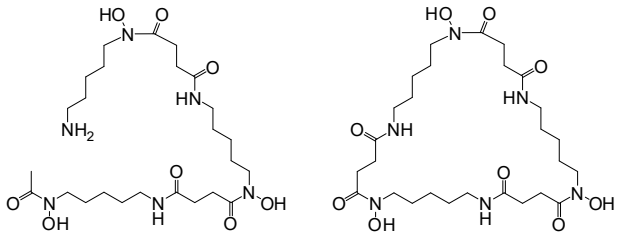

Fig. 1. Chemical structures of desferrioxamines B and E

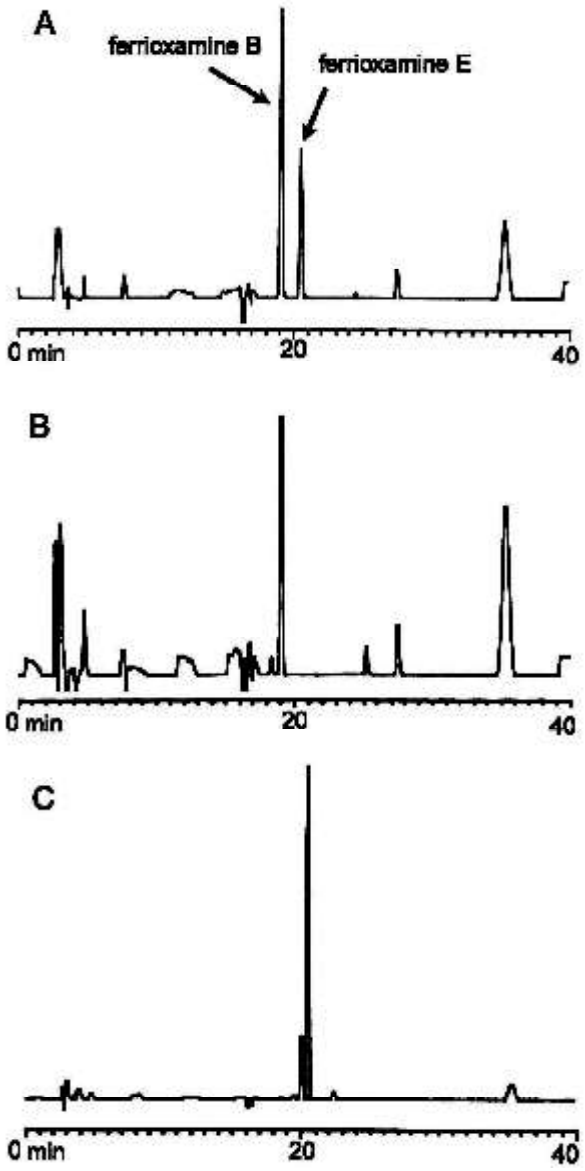

Fig. 2. HPLC chromatograms of culture media (A: *Streptomyces canus* NBRC12752; B: *S. minoensis* NBRC15797, C: *S. lydicus* NBRC13058)

Screening for siderophore using HPLC

Each bacterial strain was cultured using the shaker (180 rpm) set at 30 °C for 5 days in the conical tube containing 10 mL of the iron deficient culture medium. Each cultured medium was centrifuged at 3000 rpm, and 5 mL of the supernatant was collected into new conical tube. The 1 M $FeCl_3$ (0.05mL) was added to the supernatant to generate the ferric siderophore. After centrifugation at 10000 rpm for 5 min, 100 uL of the solution was subjected to HPLC analysis. The HPLC analysis was performed using analytical C18 column (Nacalai tesque, Cosmosil 5C18MS-II), eluted with solvent A (distilled water containing 0.05% TFA) and solvent B (MeCN containing 0.05%TFA) using time program; step1 of the isocratic mode from 0 min to 10 min with 1% solvent B, step 2 of gradient mode from 10 min to 30 min increasing elution rate of solvent B from1% to 60%. The UV/VIS detector was set at the wavelength of 425 nm. Quantification was performed using peak area on HPLC chart by comparison with authentic ferrioxamine B, which was derived from desferrioxamine B (Sigma-Aldrich).

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