Short Communication

MARINE BIOTECHNOLOGY

Isolation and Characterization of Two Groups of Novel Marine Bacteria Producing Violacein

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Abstract

Thirteen strains of novel marine bacteria producing a purple pigment were isolated from the Pacific coast of Japan. They were divided into two groups based on their 16S ribosomal RNA gene sequences, and both groups of bacteria belonged to the genus *Pseudoalteromonas*. The UV-visible spectrum of the pigment was identical to those of violacein, a pigment produced by several species of bacteria including *Chromobacterium violaceum*, an opportunistic pathogen. Further analysis of the chemical structure of the pigment by mass spectroscopy and nuclear magnetic resonance spectroscopy showed that the pigment was violacein. The high purity of violacein in the crude extract enabled us to employ simple and nonpolluting procedures to purify the pigment. Isolated bacteria may be useful as a *C. violaceum* substitute for the safe production of violacein.

Keywords: pigment — *Pseudoalteromonas* — violacein

Introduction

Bacterial pigments have often been shown to have physiological characteristics (Margalith 1992). A purple pigment, violacein, is known to be produced by the bacterium *Chromobacterium violaceum*, which was isolated from soil and water in tropical and subtropical areas (Durán and Menck 2001). Violacein has certain physiological characteristics, such as antitrypanosome and antitumor properties (Melo et al. 2000, 2003; Leon et al. 2001; Ferreira et al. 2004). These characteristics provide the possibility to use violacein for therapeutic purposes, but *C. violaceum* can also act as an opportunistic pathogen in animals and humans, and cause fatal septicemia accompanied with liver and lung abscesses (Richard 1993). Therefore, mass production of violacein using *C. violaceum* was thought to be impractical, and it is essential to search for a substitute of *C. violaceum*.

To explore bacteria producing violacein and other bioactive compounds, we isolated bacteria from the marine environment because only a few bacteria producing violet pigments have been isolated from seawater until today, and we therefore expected to find new species of bacteria and new compounds in the ocean. One of the violet pigment-producing marine bacteria is *Pseudoalteromonas luteoviolacea*, which is the sole marine bacterium producing violacein ever characterized. In Japan, several strains of *P. luteoviolacea* were isolated from Kinko Bay in Kagoshima prefecture, Japan (MacCarthy et al. 1985). *Shewanella violacea*, a deep-sea microorganism isolated from sediments of Ryukyu Trench at a depth of 5110 m, also produced a violet pigment that was recently demonstrated to be a new alkylated indigoidine (Kobayashi et al. 2007).

We isolated bacteria from seawater at a depth of 320 m off the coast of Cape Muroto, Kochi Prefecture, Japan, between 1998 and 2002 (Yada et al. 2003). Among the bacteria isolated, 13 strains of gram-negative, rod-shaped bacteria produced a violacein-like purple pigment. In the current study, we identified the isolated bacteria, characterized the chemical structure of the pigment.

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Materials and Methods

Bacterial Strains Bacterial strains, *Pseudoalteromonas denitrificans* (IAM14544) and *Pseudoalteromonas luteoviolacea* (IAM14710), were donated by the Institute of Molecular and Cellular Biosciences, the University of Tokyo.

Isolation of Bacteria The pumping facility of the Kochi Prefectural Deep Seawater Laboratory was used to sample seawater at a depth of 320 m off the coast of Cape Muroto, Kochi prefecture, Japan. Bacteria were isolated by spreading seawater on PPES-II plate medium (Taga 1968), and incubating at 20°C for 5 to 6 days.

Determination of 16S Ribosomal RNA (rRNA) Gene Sequence Bacterial genome was extracted using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) and employed as a template for polymerase chain reaction (PCR). DNA polymerase used for PCR was Ex Taq (TaKaRa Bio). The 16S rRNA gene of the bacteria was amplified by PCR, using a forward primer, 5′-AGAGTTTGATCCT GGCTCAG-3′ (corresponding to positions 8 to 27 of *E. coli* 16S rRNA gene) and a reverse primer, 5′-AAGG AGGTGATCCAGCCGCA-3′ (corresponding to positions 1542 to 1522) (Hiraishi 1992). Universal primers for the prokaryote described by Hiraishi (1992) were used to determine the sequences of the 16S rRNA gene of isolated bacteria. The homologies of the determined sequences of 16S rRNA gene were compared with sequences in GenBank database by the BLAST method (Altschul et al. 1990). The phylogenetic tree based on the determined sequences was constructed by the neighbor-joining method (Saitou and Nei 1987).

Physiological Characterization of Bacteria Denitrification activity was studied according to the method of Zimmermann and von Lengerken (1979). Optimal growth temperatures and other physiological properties of the bacteria were investigated. Growth in the presence of various carbon sources was studied by NCIMB Japan.

Production, Extraction and Purification of the Pigment Static culturing of pigment-producing bacteria was conducted in 2 L of PPES-II medium at 20°C for 10 days. The culture fluid was subsequently centrifuged at 4000 *g* for 10 min, and the pellet was resuspended with ethanol (20% volume of the culture fluid) to extract the pigment. The obtained mixture was centrifuged at 10,000 *g* for 10 min, the supernatant dried in a rotary vacuum evaporator, and ethanol (10% volume of the culture fluid) was subsequently added to dissolve the pigment. The ethanol suspension containing the pigment was centrifuged again to obtain the crude pigment.

The crude pigment was subjected to silica gel open column chromatography eluting with 2-propanol. The main fraction was subjected to octadecyl silica (ODS) open column chromatography eluting with 40% (vol/vol) acetonitrile (AcCN) aqueous solution. The blue black fraction was evaporated under reduced pressure until solid pigment remained. The pigment was harvested by centrifugation or filtration, washed successively with petroleum ether and chloroform, and dried under reduced pressure for 24 h to obtain the purified pigment. The ODS-A120-S150 resin was purchased from YMC, Kyoto, Japan.

Analysis of the Pigment High-performance liquid chromatography (HPLC) was performed on an ODS column (4.6 mm×150 mm, Beckman Ultrasphere ODS 5 μm) at a flow rate of 1 ml/min with a mobile phase of 40% (vol/vol) AcCN aqueous solution. Analyses of the pigment by electrospray ionization mass and mass/mass (ESI-MS and ESI-MS/MS) spectroscopy were conducted with a Thermo Finnigan LCQ quadrupole ion-trap mass spectrometer (Thermo, San Jose, CA). Electrospray ionization in a negative ion mode was achieved by using a spray voltage of −4.5 kV and a capillary temperature of 150°C. Flow rate of the pigment solution was 10 μl/min. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Varian Unity INOVA 400 to obtain ^{1}H (400 MHz) and ^{13}C (101 MHz) spectra of the pigment in dimethyl sulfoxide-d_6 (DMSO-d_6). Infrared (IR) spectroscopy of the pigment in a KBr pellet was conducted on a JASCO FT/IR-610.

NMR Data ^{1}H-NMR (DMSO-d_6) : 6.79 (dd, *J*=2.0, 8.5 Hz), 7.23 (d, *J*=2.0 Hz), 7.35 (d, *J*=8.5 Hz), 8.11 (d, *J*=2.5 Hz), 9.38(s, OH), 12.09 (d, NH) (5-hydroxyindole ring), 7.55 (d, *J*=1.5 Hz), 10.78 (d, NH) (2-pyrrolidone ring), 6.83 (d, *J*=8.0 Hz), 6.94 (td, *J*=8.0, 2.0 Hz), 7.19 (td, *J*=8.0, 2.0 Hz), 8.92 (d, *J*=8.0 Hz), 10.65 (s, NH) (oxindole ring) , ^{13}C-NMR (DMSO-d_6) : 104.8, 105.9, 113.2, 113.6, 125.8, 130.0, 131.8, 153.1 (5-hydroxyindole ring), 97.1, 137.2, 147.9, 171.9 (2-pyrrolidone ring), 109.2, 118.8, 121.0, 122.6, 126.5, 129.6, 142.0, and 170.4 (oxindole ring).

Results and Discussion

Identification of Pigment-Producing Bacteria Thirteen strains of marine bacteria which produced

a violacein-like purple pigment were isolated from seawater off the coast of Cape Muroto, Japan. By comparing the partial sequences corresponding to positions between 338 and 806 of *E. coli* 16S rRNA gene, pigment-producing bacteria were divided into two groups, a V1 group (520P1, 402P1, and 417P1; three strains), and V2 group (710P1, 315P1, 315P3, 315P4, 315P5, 315P11, 516P1, 612P1, 710P2, and 714P1; 10 strains). Bacteria belonging to the same group were found to have the same partial sequences, which indicated the possible presence of two species of pigment-producing bacteria. Almost complete sequences of 16S rRNA gene of the strain 520P1 (V1 group) and the strain 710P1 (V2 group) were determined (DDBJ accession numbers AB196167 and AB196168, respectively).

Based on the homology of 16S rRNA gene of bacteria, a phylogenetic tree was constructed containing the isolated bacteria (strains 520P1 and 710P1), the violacein-producing bacteria ever reported, and a related marine bacterium (*Pseudoalteromonas denitrificans*) (Figure 1). Both DNA sequences of *C. violaceum* and *J. lividum* showed low homology with those of the isolated bacteria. *P. luteoviolacea* produces violacein and deoxyviolacein, and is the only violacein-producing marine bacterium of which the pigment was ever characterized in detail (Laatsch et al., 1984). However, the 16S rRNA gene sequences of strains 520P1 and 710P1 were only 94% homologous with that of *P. luteoviolacea*. On the other hand, the DNA sequences of the strains 520P1 and 710P1 were highly homologous with that of *P. denitrificans*. *P. denitrificans* is known by its characteristic denitrification activity and production of prodigiosin (Enger et al. 1987), a red pigment with immunodepressant and antitumor characteristics (Kawauchi et al. 1997; Montaner and Perez-Tomas 2003).

Although strains 520P1 and 710P1 were closely related with *P. denitrificans*, they were different in the pigments produced, denitrification activities and utilization of carbon sources, which all indicated that they were phenotypically different bacteria from *P. denitrificans* (Table 1). Actually, *P. denitrificans* (IAM14544) cultured under the conditions described below produced prodigiosin, but not violacein. Thus it was concluded that strains 520P1 and 710P1 were novel marine bacteria which produced a violacein-like pigment. Further study, including DNA–DNA hybridization analysis, will reveal the precise phylogenetic relations between isolated strains and *P. denitrificans*.

Purification and Characterization of the Pigment

Static culture of pigment-producing bacteria was conducted in 2 L of PPES-II medium at 20°C. The pigment was produced on the surface of the medium, and 20 to 25 mg of the pigment accumulated in the culture medium after 10 days. The pigment extracted with ethanol was dried and solubilized again in ethanol to remove residual salts in the pigment. Silica gel open column chromatography was used to remove low polar compounds. Open ODS column chromatography was applied to purify the pigment effectively. Judging from the HPLC analysis on ODS column and NMR spectra, this procedure led to a highly purified pigment. Pigment was purified from the crude extract with a recovery of 40% to 50%. Violacein isolated from *P. luteoviolacea* was also purified according to the procedure described above to compare the properties of violacein with those of the pigment from strains 520P1 and 710P1.

The absorption spectrum of the pigment with maximum absorbance at 575 nm in ethanol was identical to that of violacein from *P. luteoviolacea*. On HPLC analysis, the pigment and violacein

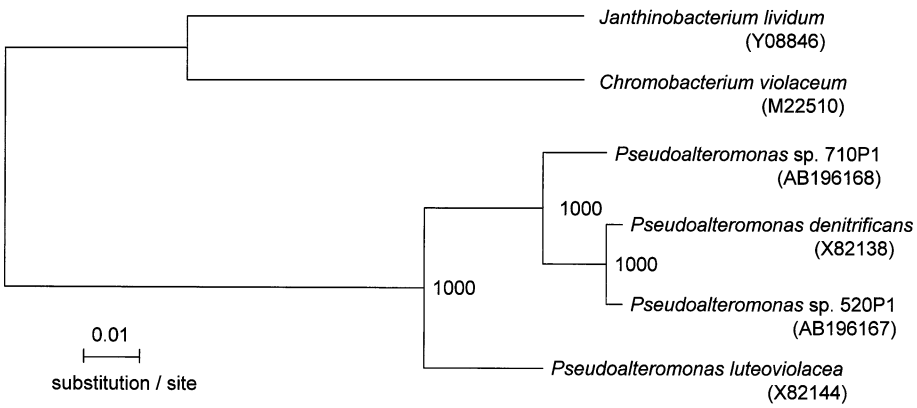

Figure 1. Phylogenetic relationships of strains 520P1 and 710P1 to violacein-producing bacteria. The DNA sequences of 16S rRNA gene in DDBJ and GenBank were used for comparison. The numbers in parentheses are accession numbers in the DDBJ and GenBank databases.

Table 1. Characteristics of the bacterial strains

Strain	*520P1*	*710P1*	*P. denitrificans*	*P. luteoviolacea*
Gram staining	–	–	–	–
Growth at	10–25°C	10–25°C	4–20°C	15–37°C
Reduction of NO^{3-} to NO^{2-}	–[b]	+	–	–
Denitrification with gas formation	–	–	+	–
Colony pigmentation:	Violet	Violet	Red	Violet
Utilization of:[a]				
D-Glucose	–	–	+	+
D-Mannose	–	–	ND	ND
Maltose	–	–	+	+
D-Gluconate	–	–	–	–
N-Acetylglucosamine	–	–	–	+
Citrate	–	–	ND	–
D-Mannitol	–	–	–	–

[a]Data for *P. denitrificans* and *P. luteoviolacea*: Bergey (1994).
[b]Bacteria of the V1 group, except strain 520P1, showed the ability to reduce NO^{3-}.
ND, not determined.

showed the same elution profiles. The pigment from strain 520P1 was further analyzed by ESI-MS and NMR spectroscopy. In the negative-ion ESI-MS spectrum of the pigment, a quasimolecular ion peak was observed at *m/z* 342.5 $[M–H]^-$. The MS/MS spectrum of the ion at *m/z* 342.5 showed the fragment ions at *m/z* 298.4, *m/z* 209.3 and *m/z* 157.3. Moreover, violacein from *P. luteoviolacea* gave the same molecular ion and fragment ions, which indicate that both the pigment and violacein have the same molecular formula ($C_{20}H_{13}O_3N_3$), and share the same chemical structural backbone.

By analyzing the data of ^{1}H-NMR and ^{13}C-NMR spectra, it is apparent that the pigment is highly purified violacein. In the ^{1}H-NMR spectrum, prominent peaks were observed in the low magnetic field. Three sharp N-H signals (δ 12.09, δ 10.78, and δ 10.65) were the most striking features of violacein (Laatsch et al. 1984; Hoshino et al. 1987; Rettori and Durán 1998). The signal at δ 9.38 was assigned to the proton of OH. The ^{13}C signal at δ153.1 also indicates that the pigment was violacein, since the signal of the corresponding carbon of deoxyviolacein which lacks a hydroxyl residue appears in the higher magnetic field (Hoshino et al. 1987). Assignments of all the ^{1}H and ^{13}C signals were established by nuclear Overhauser enhancement spectroscopy (NOESY), total correlation spectroscopy (TOCSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC) experiments. The pigment from strain 710P1 was also shown to be violacein by ^{1}H- and ^{13}C-NMR spectroscopy. The IR spectrum of the pigment showed absorption bands of carbonyl bonds around 1600 cm^{-1}, which also coincided with the features of violacein (Rettori and Durán 1998).

In the present study, we characterized novel bacterial strains and the produced pigment which was shown to be violacein. As far as we know, this is the first demonstration of bacteria producing violacein from the marine environment below a depth of 300 m. Although several species of bacteria produce violacein, violacein for research and therapeutic purposes has been usually obtained from a culture of *C. violaceum*. However, the pathogenicity of *C. violaceum* in human and animals is an inevitable obstacle for the mass production of violacein. On the other hand, the marine bacteria characterized here could not survive at 37°C, the temperature of the human body. Therefore, they are considered to be nonpathogenic and suitable for mass production of violacein. Further investigations in the production of violacein are still required to achieve mass production of the pigment in the future.

P. luteoviolacea is another marine bacteria producing violacein. However, *P. luteoviolacea* (IAM14710) cultured in this study produced pigments other than violacein and deoxyviolacein, and these unidentified pigments still remained after silica gel chromatography. Yellow pigments produced by *P. luteoviolacea* are also described by MacCarthy et al. (1985). On the other hand, bacteria we isolated only produced violacein and a very small amount of pigment assumed to be deoxyviolacein. Therefore, after chromatography using silica gel with a mobile phase of 2-propanol, almost all the pigment obtained was violacein. Thus a higher purity of violacein in the crude extract enabled us to use simple and nonpolluting procedures of purification, avoiding harmful organic solvents.

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