論 文 内 容 の 要 旨

INTRODUCTION

Bacterial pigments have received much attention in recent years because they show better biodegradability and therefore have higher compatibility with the environment (Venil *et al.*, 2013). Violacein, a purple pigment which is produced by several species of terrestrial and marine bacteria, is a secondary metabolite that possesses various biological properties, such as antibacterial, antiviral, antitrypanosomal, and antitumor activities (Hoshino, 2011; Durán and Menck, 2001). These bioactive properties offer the possibility to use violacein for medical applications.

Until now, it has been reported that violacein can be produced by various Gram-negative bacteria, including *Chromobacterium violaceum* (Durán and Menck, 2001), *Janthinobacterium lividum* (Shirata *et al.*, 2000; Pantanella *et al.*, 2007), *Pseudoalteromonas luteoviolacea* (Gauthier, 1982; McCarthy *et al.*, 1985), and *Pseudoalteromonas* sp. 520P1 (Yada *et al.*, 2008).

Studies on the biosysthesis of violacein have been conducted to reveal the components involved the biosynthetic pathway. It has been reported that five enzymes, VioA to VioE, are responsible for the production of violacein in *Chromobacterium violaceum* (August *et al.*, 2000). VioE is demonstrated to play an important role in synthesizing of the molecular skeleton of violacein (Hirano *et al.*, 2008). These enzymes are encoded by five genes, *vioA* to *vioE* (Momen and Hoshino, 2000). These five genes

are also identified to exist within a single operon, that is, vioABCDE (August *et al.*, 2000).

The expression of the vioABCDE operon is known to be regulated by a quorum sensing mechanism in *Chromobacterium violaceum* via the response to small signaling molecules, generally called as *N*-acylhomoserine lactones (AHLs) (McClean *et al.*, 1997; Morohoshi *et al.*, 2010). The AHLs are produced and secreted during bacterial growth. When the concentration of AHLs reach a threshold level (quorum level), it induces phenotypic effects by regulating quorum sensing-dependent target gene expression. It has been proven that the quorum sensing system in *Chromobacterium violaceum* contains two components. The first is an AHL synthase (usually LuxI or LuxI homolog) which is responsible for AHL synthesis. The second one is a regulatory protein (LuxR or LuxR homolog) which generally promotes transcription of the target gene, when bound with AHL. LuxI/LuxR quorum sensing system was found for the first time in a marine bioluminescence bacterium *Vibrio fisheri* (Antunes *et al.,* 2010). At present, more than 70 different Gram-negative bacteria have been reported to contain the LuxI/LuxR-type quorum sensing system (Czajkowski and Jafra, 2009).

In recent studies, Wang *et al.* (2008) reported that the production of violacein by *Pseudoalteromonas* sp. 520P1, a Gram-negative marine bacterium isolated from the Pacific coast of Japan, was also under the regulation of an AHL-dependent quorum sensing. The gene cluster responsible for violacein biosynthesis in *Pseudoalteromonas* sp. 520P1 has also been characterized. A cluster of five ORFs (vioABCDE) of the length of 7383 bp has been cloned. However, the *luxI* gene and *luxR* gene in strain 520P1 have not been identified so far. Identification of these genes is crucial to extend our

knowledge of the whole regulatory mechanism of the violacein biosynthesis in *Pseudoalteromonas* sp. 520P1.

In this study, we conducted whole genome analysis of *Pseudoalteromonas* sp. 520P1 to identify the *luxI* and *luxR* genes and expressed the cloned *luxI* in *Escherichia coli*.

MATERIAL AND METHODS

Bacterial strains, plasmids, and reagents

Genomic DNA of *Pseudoalteromonas* 520P1 No.412 strain was used to sequence the whole genome and cloning of the *luxI* gene. This strain was deposited into the NITE Biological Resource Center (NBRC) and assigned the accession numbers NBRC 107704. *Agrobacterium tumefaciens* NTL4 (pZLR4) was used as a reporter strain to detect AHLs.

Plasmid pUC18 and *Escherichia coli* DH5α were purchased from Takara Bio (Otsu, Japan). Plasmid pET28a (+) and *Escherichia coli* BL21 (DE3) were purchased from Novagen (Darmstadt, Germany). KOD-plus DNA polymerase for PCR amplification was purchased from Toyoba (Osaka, Japan), Restriction enzymes, a DNA ligation kit (Mighty Mix), X-gal and IPTG were purchased from Takara Bio. Ampicillin (100 μg/ml), kanamycin (50 μg/ml) was used for the selection of recombinant *E. coli*.

GenBank/DDBJ and NBRC accession numbers

The accession numbers of protein sequences in DDBJ/EMBL/GenBank used in this study were P54656 (EsaI, *Pantoea stewartii* subsp. *stewartii*); YP_659946 (LuxI, *Pseudoalteromonas atlantica* T6c); ERG18256 (LuxI, *Pseudoalteromonas citrea* NCIMB

1889), L8D0L3 (LuxR, *Pseudoalteromonas luteoviolacea* B, ATCC 29581); YP_ 659944 (LuxR, *Pseudoalteromonas atlantica* T6c). Homologous sequences were multiply aligned using CLUSTALW (Thompson et al., 1994) followed by a manual annotation.

Genomic sequence and analysis of strain 520P1 No.412

Genomic DNA of strain 520P1 No. 412 was purified using a QIAGEN Genomic DNA kit with a Genome-tip 100/G column (Qiagen KK, Tokyo, Japan). The genome was sequenced on an Illumina Hiseq 2000 system by Macrogen Japan (Tokyo, Japan). A total of 5,740,346 reads were assembled using SOAPdenovo Assembly into 67 scaffolds with an N_{50} length of 136,339bp.

Cloning of luxI gene

Based on the sequence of *luxI* gene of scaffold 19 in strain 520P1, a set of primers was designed and used to amplify the DNA region containing *luxI* gene by the first and the second PCR. The genomic DNA of *Pseudoalteromonas* sp. 520P1 No. 412 was used as the template for the first PCR. Then, PCR product was purified and used as the template for the second PCR. The PCR product of the second PCR containing restriction enzymes on both ends was cloned into the multiple cloning site of pET28a(+) linearized with the restriction enzymes *Nde*I and *Hind*III. The recombinant plasmid was transformed into competent *E. coli* BL21 (DE3) by heat shock method. All PCR were performed with fidelity KOD-Plus DNA polymerase. PCR products were purified using a PCR purification kit or gel extraction kit (Qiagen KK, Tokyo, Japan).

Expression of luxI gene

E. coli BL21 (DE3) harboring the recombinant plasmid was inoculated into 200-ml flasks containing 50 ml fresh LB medium. The flasks were incubated on a shaker at

37°C until the optical density at 600 nm (OD_{600}) of the cultures reached 1.0. Then, the flasks were transferred to 20°C incubator and AHLs production was carried out in the presence and absence of 0.5 mM isopropyl ß-D-thiogalactopyranoside (IPTG). AHLs were extracted from the culture supernatant and measured as described below.

Extraction of AHLs

After 24h and 48h of incubation at 20°C with shaking (180 rpm), the culture supernatants were harvested by centrifugation at 8,000 rpm (11,800 x g) for 30 min. Then, AHLs in the supernatants were extracted with the same volume of ethyl acetate (AcCN) in a separating funnel. The ethyl acetate phases were recovered and evaporated to dryness. The dried samples were dissolved in 1.0 ml of ethanol. The extracts were stored at -20°C until use.

Plate assay of AHLs

Agrobacterium tumefaciens NTL4 was cultured in AB medium containing 0.2% glucose and 1 mg/L gentamicin on shaker at 200 rpm at 28ºC. An AB minimal agar plate containing 0.2% glucose and 40 mg/ml of 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) was overlaid with 3ml of molten soft agar containing 40 mg/ml of X-Gal and 200 µl of the overnight culture of *Agrobacterium tumefaciens* NTL4 (pZLR4). After the soft agar was solidified, paper discs (8 mm in diameter) filled with 20 µl of AHL extract were laid on the surface of the plate. Following incubation for 24–48 h, the plate was examined for zones of blue pigmentation (Wang *et al.*, 2008).

TLC assay of AHLs

Reverse-phase (RP) – TLC plates were used to determine type of AHLs. The AHL extracts were spotted onto a TLC plate (Silica Gel 60 RP-18F_{254}S Merck, Darmstadt, Germany), and the plate was developed in a solvent system of methanol:water (60:40, v/v). After chromatography, molten soft agar containing 0.2% glucose, 40 μg/ml of X-Gal and 1/15 volumes of overnight culture of *Agrobacterium tumefaciens* NTL4 (pZLR4) was poured over the surface of the TLC plate, and the plate was left until the soft agar was solidified. Then, the TLC plate was incubated at 28^0C for 24–48 h in a closed plastic container (Shaw PD *et al.*, 1997; Wang *et al.*, 2008).

RESULTS

At first, based on the information on the nucleotide sequences of *luxI* homologs from other *Pseudoalteromonas* strains, we tried to amplify the *luxI* gene in strain 520P1 by PCR. However, probably because of low homology between nucleotide sequences of the superfamily of *luxI* gene, *luxI* homolog of strain 520P1 has not been amplified successfully.

As an alternative, we tried to sequence the whole genome of *Pseudoalteromonas* strain 520P1 no.412 (NBRC 107704) to find the *luxI* and *luxR* genes. The assembled draft genome sequence of 67 scaffolds with the size of approximately 5.25Mb was analyzed to locate quorum-sensing-related genes. A total of 4,899 protein-coding regions and 99 RNA-coding sequences were detected using the GLIMMER system and RAST Server respectively. Annotation and mapping using BLAST and the RAST server revealed that at least one pair of *luxI/luxR* gene was located on the scaffold 19 and four *luxR* homologs were located on different scaffolds of strain 520P1 No. 412. The orientation of the *luxI* and *luxR* genes on the scaffold 19 was in the opposite direction.

The genome of strain 520P1 No.412 has been deposited in DDBJ/EMBL/GenBank under the accession number BBIN01000000.

To examine the ability of the *luxI* gene to produce AHLs *in vivo*, heterologous expression of the *luxI* gene was conducted in *E. coli* using a recombinant pET vector. The *luxI* gene was amplified from the genomic DNA of *Pseudoalteromonas* sp. 520P1 No. 412 by nested PCR. The amplified gene was cloned into pET-28a(+) and expressed in *E. coli* BL21 (DE3). AHLs were extracted from culture supernatant and detected by plate assay of AHL using *Agrobacterium tumefaciens* NTL4 (pZLR4) as an AHL-reporter strain. Further analysis of the AHL extract by TLC revealed two spots of AHLs on the TLC plate, indicating that the extract contained two types of AHLs. Our results revealed that the *luxI* gene was successfully cloned into the vector and expressed in *E. coli*. In the plate assay, *A. tumefaciens* NTL4 (pZLR4) detected AHL activity in the culture extract of a recombinant *E.coli* containing the *luxI* gene from the DNA (scaffold 19) of strain 520P1.

DISCUSSION

In this study, the result of AHL assay suggested that at least one *luxI* from strain 520P1 encoded an enzyme with AHL synthase activity when expressed in *E. coli* BL21 (DE3). The result of TLC analysis showed that at least two types of AHLs existed in the extract from *E. coli* containing the luxI of scaffold 19. Further studies are needed to reveal the involvement of the *luxI* gene in the regulation of violacein production in *Pseudoalteromonas* sp. 520P1.