Characterization of *Massilia* sp. BS-1, a Novel Violacein-producing Bacterium Isolated from Soil

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Massilia sp. BS-1, a novel bacterium, was characterized from a view point of violacein production. During cultivation, strain BS-1 began to produce violacein in the early stage of log phase growth at an incubation time of 16 h, and the amount of violacein rapidly reached a maximum value of 62 mg/L for 5 h. This observation was suggested that the violacein production was regulated by quorum sensing because it was not growth-associated and occurred over a short period. Strain BS-1 did not produce violacein in a synthetic MM2 medium without L-histidine. Neither *N*-hexanoyl-DL-homoserine lactone nor *N*-(3-oxooctanoyl)-DL-homoserine lactone, known as autoinducers of quorum sensing, induced violacein production of strain BS-1. Any acetyl homoserine lactones were not detected in the culture broth by LC-MS analysis. However, the supernatant of the culture broth of strain BS-1 induced violacein production.

Key word: *Massilia*, Violacein, Quorum sensing, Autoinducer

1. Introduction

Violacein is a bluish-purple pigment that has various important biological activities including antibacterial (against gram-positive bacteria)[1], antifungal[2], anti-protozoan[3], anti-malarial[4], anti-tumor[5], anti-viral[6], anti-oxidant[7], and anti-diarrheal[8] activities. The violacein carbon skeleton is derived from 2 molecules of L-tryptophan (Fig. 1). Although further investigation to elucidate the pharmaceutical potential of violacein is needed, mass production of violacein is difficult due to its low productivity. The role of violacein production in its producers has not been understood, but it is suggested that it provides a survival advantage to species over other microorganisms in the environment.

It has been reported that various bacteria, such as *Chromobacterium violaceum*[9], *C. fluviatile*[9], *Janthinobacterium lividum*[10], *Alteromonas luteoviolacea*[11], *Pseudoalteromonas luteoviolacea*[12], *Duganella* sp.[13], and *Collimonas* sp.[14], produce violacein. We have already reported that a novel bacterium, *Massilia* sp. BS-1, was isolated from a soil sample and produced violacein and deoxyviolacein[15].

The production of violacein is known to be regulated via quorum-sensing molecules, for example, *N*-hexanoyl-L-homoserine lactone (C6-HSL) in *C. violaceum*[16] and *N*-(3-oxooctanoyl)-L-homoserine lactone in *Pseudoalteromonas* sp. 520P1[17]. Among the Gram-negative bacteria, *N*-acylhomoserine lactones (AHLs) are common quorum sensing molecules (autoinducers). Quorum sensing allows a species to measure its population density and control gene expression in a population density-dependent manner. It is now clear that quorum sensing is the norm

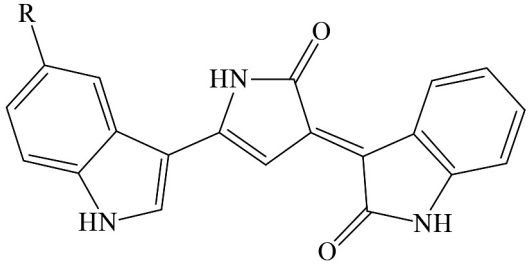

Fig. 1. Chemical structures of violacein (R＝OH) and deoxyviolacein (R＝H).

in the bacterial world and that this process is fundamental to microbiology. Therefore, it is very important to discover new producers of violacein to effectively produce it and to elucidate the mechanism of quorum sensing.

In this study, we characterize *Massilia* sp. BS-1 from a view point of violacein production and examine whether the violacein production is regulated by quorum sensing.

2. Materials and methods

Bacterial strain and growth conditions *Massilia* sp. BS-1 was isolated in our laboratory[15]. Strain BS-1 was cultivated on a nutrient agar plate containing 0.1% yeast extract, 0.1% polypeptone, 0.1% KH_2PO_4, and 1.5% agar (pH 6.8). The agar plate was incubated at 28℃ for 2-3 days. The strain was also cultivated in nutrient broth consisting of the same components described above without agar at 28℃ for 1-2 days with shaking. A synthetic MM2 medium containing 0.2% glucose, 0.1% $(NH_4)_2SO_4$, 0.4% $Na_2HPO_4{\cdot}7H_2O$, 0.2% KH_2PO_4, and 0.01% $MgSO_4{\cdot}7H_2O$ was used for the characterization of strain BS-1. *N*-Hexanoyl-DL-homoserine lacton and *N*-tetradecanoyl-DL-homoserine lactone were purchased and used to induce violacein production as AHLs.

Production of violacein To produce violacein, strain BS-1 was cultivated in 30 mL of nutrient broth on a rotary shaker at 28℃ for 18 h. Thirty milliliters of the seed culture was transferred to a 3-L jar fermentor containing 2.0 L of nutrient broth followed by cultivation with aeration (0.5 v/v/m) and agitation (300 rpm) at 25℃. Samples (3.0 mL) withdrawn from the culture broth at regular intervals were used for measuring pH, bacterial growth, and violacein production. Violacein was harvested from the sample by centrifugation, extracted by methanol (3.0 mL), and quantified using a spectrophotometer at 577 nm. Bacterial growth was monitored by measuring the turbidity at 660 nm. After 67 h of cultivation, the cells were centrifuged at 8,000 × *g* for 10 min, and the supernatant was discarded. The cell pellet was then mixed with 200 mL of methanol. The mixture of the cells and methanol was treated by ultrasonication until the cells were completely bleached. The methanol extract was then separated from the cells by centrifugation at 8,000 × *g* for 10 min. The extract was concentrated to remove methanol in a rotary vacuum evaporator and was extracted twice with an equal volume of ethylacetate. The extract was evaporated under vacuum to obtain a crude mixture of violacein and deoxyviolacein.

Quantitative and qualitative analysis The amount of violet pigment, consisting of violacein and deoxyviolacein, in the culture broth was estimated using their molecular absorption coefficient ($\varepsilon = 1.7 \times 10^4$ M^{-1} cm^{-1}, $\lambda = 577$ nm)[19]. Quantitative analysis of the pigment was performed using high performance liquid chromatography (HPLC) with a ZORBAX SB-Aq column (150 × 4.6 mm i.d.) (Agilent Technologies, Inc., USA) at 577 nm and 40℃ with MeOH / water (70:30 [V/V]) as the mobile phase at a flow rate of 0.5 mL/min. Qualitative analysis of the culture broth was carried out by liquid chromatography-mass spectrometry (LC-MS). The analysis was performed using a time of flight mass spectrometer (Xevo QTof MS, Waters Corp., USA). HPLC separation was performed using an ACQUITY UPLC BEH C18 1.7μm column (50 × 2.1mm i.d.) at 40℃ with a mixture of water and acetonitrile contained 0.1% formic acid as the mobile phase at a flow rate of 0.2 mL/min. To detect trace amounts of AHLs in the culture broth, we used the selected reaction monitoring technique. The characteristic fragment ion of m/z 102, derived from the homoserine lactone moiety of the AHLs, was monitored selectively to detect all kinds of AHLs.

Antibacterial activities of violacein The growth inhibitory and lethal effect of the violet pigment, a mixture of violacein and deoxyviolacein, isolated from strain BS-1 were examined for several bacteria such as *Bacillus subtilis*, *Staphylococcus aureus* and E*scherihia coli* using the standard methods[20].

Assay for autoinducers of violacein To determine whether autoinducers are produced by strain BS-1, we attempted the following assay (Fig. 2): Strain BS-1 was cultivated in

nutrient broth with shaking as described above. Two ml of the culture broth was sampled at various times for the cultivation time from 6 h to 19 h, and was centrifuged to get a supernatant. The supernatant was assayed for autoinducer activity. The autoinducer activity showed the violacein production induced by autoinducers in the supetnatant.

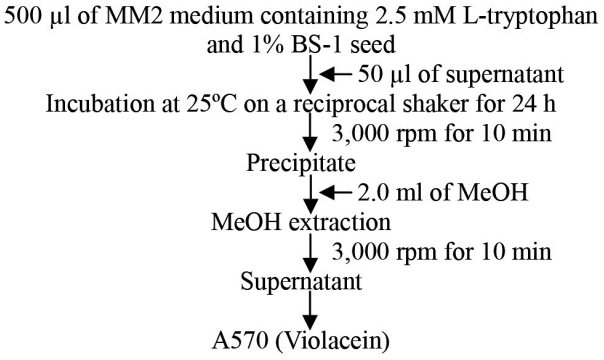

Fig. 2. Assay for autoinducers of violacein production.

3. Results and Discussion

Cultivation of strain BS-1 *Massilia* sp. BS-1 is a rod-shaped Gram-negative bacterium that exhibits motility and flocculation. On a nutrient agar plate, it formed a violet colony whose surface was wavelike and hard. Although it is an obligate aerobe, it grew under sessile conditions, forming extended biofilms on the surface of the culture broth (Fig. 3).

However, strain BS-1 did not produce violacein in a synthetic MM2 medium, even if L-tryptophan was added, with under aerobic conditions, although the strain grew well and produced biofilms (Fig. 4).

Time courses for growth and production When strain BS-1 was cultivated in the nutrient broth with shaking, it grew to stationary phase and ended violacein production by an incubation time of 24 h. It began to produce a bluish-purple pigment in the early period of log phase growth at an incubation time of 16 h, and production rapidly reached a maximum value of 62 mg/L pigment at 5 h (Fig. 5). The color of the culture changed to a deep violet due to the pigment. It appeared

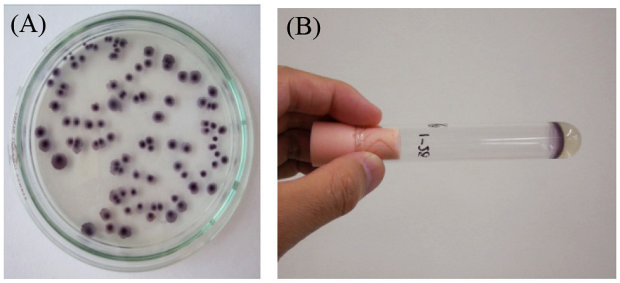

Fig. 3. Cultures on an agar plate (A) and formation of biofilm (B).

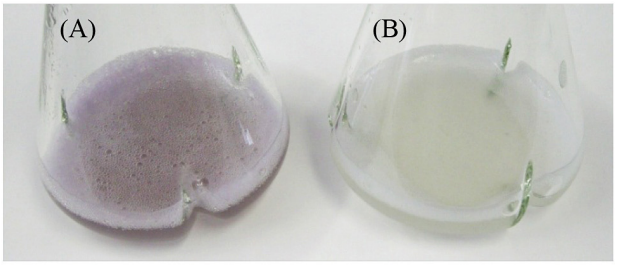

Fig. 4. Non-production of violacein in a synthetic MM2 medium (B).
Strain BS-1 was cultivated in nutrient medium (A) and MM2 medium (B) supplemented with 1.0 mM L-tryptophan under the same conditions.

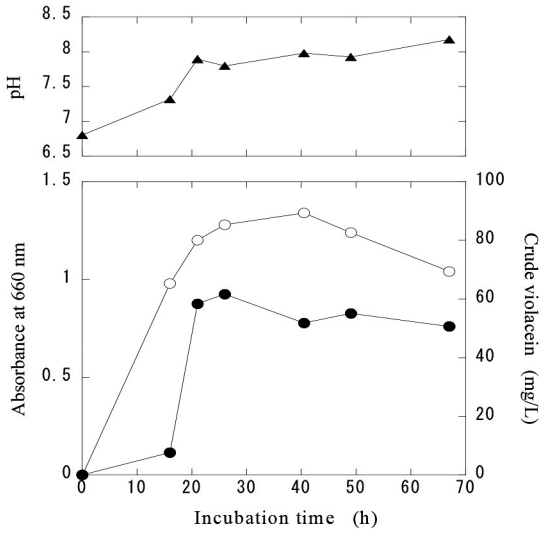

Fig. 5. Time course profile of *Massilia* sp. BS-1 cultivated in nutrient broth.
Symbols: (◆) OD660; (●) crude violacein concentration; (▲) pH.

that pigment production was regulated, because it was not growth-associated and occurred over a short period.

Characterization of the pigment The pigment

is water-insoluble and is present in the extracellular polymers produced by strain BS-1. The pigment was separated into two components with maximum absorption wavelengths of 577 nm and 555 nm by preparative HPLC. Under analytical HPLC conditions, the former (violacein) and the latter (deoxyviolacein) components were eluted at 5.51 min and 7.82 min respectively (Fig. 6).

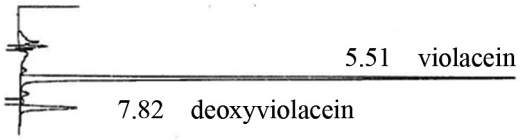

Fig. 6. HPLC analysis of the extract of the culture broth. HPLC conditions are described in Materials and Methods.

The violacein concentration was about 10-fold higher than that of deoxyviolacein. The molecular masses (*m/z*) of these two components were determined to be 344.24 (positive) and 328.23 (positive) respectively by LC-MS analysis. The entirety of the carbon, hydrogen, and nitrogen skeleton of violacein is derived from 2 molecules of L-tryptophan, and the 3 oxygen atoms originate from molecular oxygen[2].

Antibacterial activities of violacein The antibacterial effect of the violet pigment containing violacein and deoxyviolacein was confirmed for putrefactive bacteria such as *B. subtilis*, *S. aureus* and *E. coli*. The minimum inhibitory concentration (MIC) of the violet pigment for *B. subtilis* and *S. aureus* was 2.5 mg/L. MIC is defined as the lowest concentration of antibacterial material that can inhibit cell growth completely. The concentration of the violet pigment more than 20 mg/L caused lethal effects on *B. subtilis* and *S. aureus*. The lethal effect means the death of logarithmically growing bacterium. However, the violet pigment could not inhibit the growth of *E. coli* even if more than 30 mg/L.

Effect of L-histidine on violacein production In *C. violaceum*, the production of violacein is under the control of signal molecules called autoinducers[16]. The signal molecules are detected by cognate cytoplasmic receptors, and then the activated receptors bind DNA and induce transcription of target quorum-sensing genes.[20] Therefore, we considered that strain BS-1 could not produce autoinducers for violacein production and tried to determine which amino acids were necessary for violacein production in a synthetic MM2 medium. We found that L-histidine was necessary for violacein production (Fig. 7A). When strain BS-1 was cultivated in MM2 medium containing 0.04 mM L-histidine and various amounts of L-tryptophan (0-5 mM), violacein production increased with the increase in L-tryptophan till it reached a value of 0.38 mM violacein (Fig. 7B). These results suggest that L-histidine is necessary for the production of the autoinducer because L-histidine is not the source of violacein.

Putative quorum sensing in *Massilia* sp. BS-1 In Gram-negative bacteria, autoinducers are generally acylhomoserine lactones (AHLs) that differ in the structure of their *N*-acyl side chains.[21]

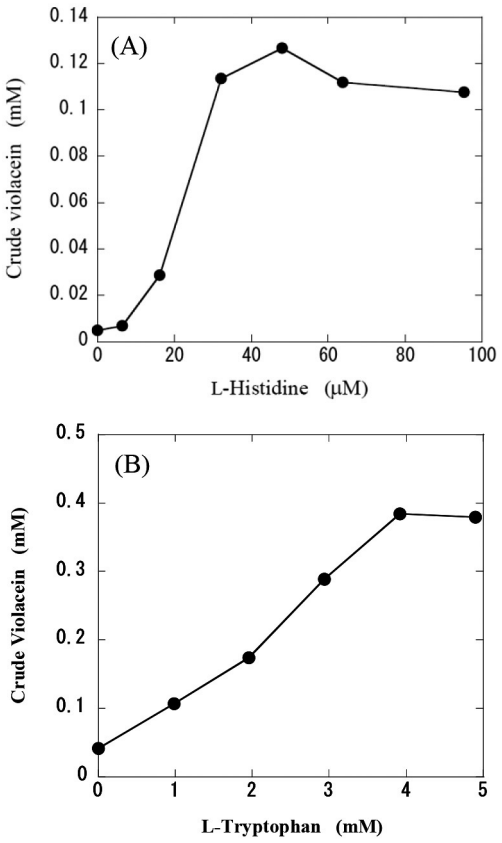

Fig. 7. Effect of L-histidine (A) and L-tryptophan (B) on violacein production by strain BS-1 grown in MM2 medium in the presence of 1.0 mM L- tryptophan (A) or 0.04 mM L-histidine (B).

However, strain BS-1 did not produce violacein in MM2 medium containing 0.1 mM *N*-hexanoyl-DL-homoserine lacton or 0.1 mM *N*-tetradecanoyl-DL-homoserine lactone. Moreover, to determine whether AHLs are produced by strain BS-1, we attempted to extract autoinducers by ethylacetate from a culture broth sampled just before violacein production. Strain BS-1 was subsequently cultivated in MM2 medium containing both L-tryptophan and the concentrated extract for 2 days. However strain BS-1 did not produce violacein. These results showed that autoinducers of strain BS-1 were not hydrophobic compounds like AHLs. We also used LC-MS to detect AHLs in the culture broth of strain BS-1. To identify the selected ions as AHLs, the characteristic fragment ion of m/z 102, derived from the homoserine lactone moiety of the AHLs, was monitored selectively. A fragment ion of m/z 102 was not detected in the sample from the culture extract. Gram-positive quorum-sensing bacteria, such as *Streptococcus* and *Bacillus*, predominantly communicate with short peptides that often contain chemical modifications[20]. The autoinducer of strain BS-1 might be a hydrophilic compound like peptide. So we assayed the autoinducer activity in the culture broth of strain BS-1 sampled at various times for the cultivation time from 6 h to 19 h. We detected the autoinducer activity in the early stages of violacein production (Fig. 8). The peak of the autoinducer activity was after 10 hours of the cultivation time, and the activity was decreased rapidly as the violacein production progressed.

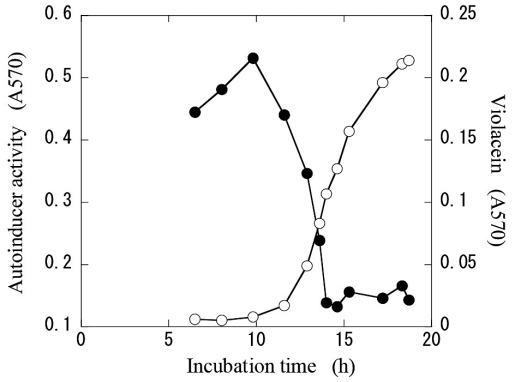

Fig. 8. Autoinducer activity in the early stages of violacein production.
Symbols: (●) Autoinducer activity (A570); (○) Violacein (A570).

The role of L-histidine and the elucidation of chemical structure of autoinducer in violacein synthesis in *Massilia* sp. BS-1 are currently under investigation.

4. Conclusion

1) *Massilia* sp. BS-1 produced a bluish-purple pigment and biofilms in nutrient medium. The pigment consists of violacein and deoxyviolacein.
2) *Massilia* sp. BS-1 grew well in a synthetic MM2 medium, while both L-tryptophan and 40μM L-histidine were neede to produce violacein. L-Tryptophan is a building block of violacein. However, the role of L-histidine is unknown.
3) Neither *N*-hexanoyl-DL-homoserine lacton nor *N*-tetradecanoyl-DL-homoserine lactone induced violacein production. Any acetyl homoserine lactones were not detected in the culture broth by LC-MS analysisi. However, the supernatant of the culture broth of strain BS-1 induced violacein production.

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