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ORIGINAL PAPER

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New violet 3,3′-bipyridyl pigment purified from deep-sea microorganism *Shewanella violacea* DSS12

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Abstract We have purified a new violet pigment derived from *Shewanella violacea* DSS12 to determine its chemical structure. The pigment colored blue in tetrahydrofuran (THF) or chloroform and showed a broad absorption spectrum from 500 to 700 nm. X-ray diffraction analysis of single crystals showed that the chemical structure of this pigment was 5,5′-didodecylamino-4,4′-dihydroxy-3,3′-diazodiphenoquinone-(2,2′), containing the same chromophore as an indigoidine known as microbial blue pigment. The violet color of this pigment was due to hypsochromic shift (blue shift) caused by the side-by-side orientation of this pigment molecule, revealed by X-ray structural analyses of a single crystal.

Keywords *Shewanella violacea* · Indigoidine · Deep-sea · Violet pigment · Crystal structure

Introduction

Recently, deep-sea environments are being focused as resources for the discovery of novel or ancient microorganisms (Massana et al. 2004; Todo et al. 2005). We have also isolated several microorganisms from various deep-sea environments, including hydrothermal vents, cold seeps and the Mariana Trench, Challenger-deep, the deepest site in the world (Takami et al. 1999; Inagaki et al. 2003; Takai et al. 2003). *Shewanella violacea* (*S. violacea*) strain DSS12 (JCM10179^T) is one of these deep-sea microorganisms, isolated as a piezo(baro)philic and psychrophilic bacterium from the deep-sea sediment of the Ryukyu Trench at a depth of 5,110 m (Nogi et al. 1998). Strain DSS12 can grow at 4–12°C and its optimum hydrostatic pressure for growth is 30 MPa, very close to its natural habitat (Kato et al. 2001). Colonies of strain DSS12 are violet in color and thus have been given the name "*violacea*". Violacein has been reported as a bacterial violet pigment produced by *Chromobacterium violaceum* (*C. violaceum*), *Janthinobacterium lividum* or *Pseudoalteromonas luteoviolacea* (Kimmel 1968; Gauthier 1975). Violacein is a dimeric structure composed of 5-hydroxyindole, oxindole and 2-pyrolidone subunits formed by the condensation of two modified tryptophan molecules and shows some biological activities such as antibacterial activity and cytotoxicity (Gauthier et al. 1975; Momen et al. 2000; Leon et al. 2001). Its synthetic gene cluster was also characterized from a DNA sequence of *C. violaceum* (August et al. 2000).

In preliminary experiments, the violet pigment produced by *S. violacea* DSS12 showed different properties from violacein, such as the solubility in methanol. *S. violacea* DSS12 produces new violet pigments other than violacein. In this study, we purified and crystallized the violet pigment from DSS12 strain to determine its chemical structure. The violet pigment produced by DSS12 was not violacein or chemically modified violacein, but a new alkylated indigoidine, which is a blue pigment produced by phytopathogens, *Erwinia chrysanthemi* (*E. chrysanthemi*), *Clavibacter michiganensis* and other saprophytic microorganisms such as *Arthrobacter atrocyaneus* and *Vogesella indigofera* (Kuhn et al. 1965; Starr et al. 1966).

Materials and methods

Strain, media and culture condition

Shewanella violacea DSS12 (JCM10179^T) was grown in Marine broth 2216 (Difco, Detroit, MI) (abbreviated

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MB). For production of violet pigment we used modified MB, containing additional 0.3%(w/v) of yeast extract [final 0.5% (w/v)] and 0.2% (w/v) of casamino acid. To prepare solid media for cultivation of bacteria, the media were supplemented with 1.5% (w/v) agar. Strain DSS12 was cultivated in MB for 3–4 days at 8°C. Cells were harvested (5,000 rpm, 20 min, 4°C) and suspended in fresh MB. Cell suspensions were spotted on the modified MB agar and incubated at 8°C for 4 days.

Extraction and purification of violet pigment from *S. violacea* DSS12

Violet pigments were extracted using chloroform–methanol (1:1) and chloroform from freeze-dried cell grown on MB agar. After evaporating the solvent, violet pigment was washed with *n*-hexane and methanol three times, and then re-suspended in chloroform. Violet pigment was purified using a silica gel column [(10 × 250 mm, silica gel 60 (70–230 mesh), Nacalai Tesque, Kyoto, Japan]. The pigment fraction was gathered and washed with double distilled H_2O, then dissolved in tetrahydrofuran (THF) and crystallized by lowering the temperature of the saturated solution from 60 to 20°C for 3 days. Impurities were checked by thin layer chromatography using silica gel plate and gas chromatography. Solubility of this pigment was measured by suspending into 100 ml of various solvents. All solvents were evaporated after removing insoluble pigments by filtration. The amount of remaining pigment was measured at A_{616} after being re-dissolved in THF.

Gas chromatography-mass spectrometry (GC-MS)

The molecular weight was measured by GC-MS (GCMS-QP5050, Shimazu, Tokyo). GC-MS was performed by the electron ionization and direct injection mode. When only mass spectrometry was used, the sample was injected into the ionization chamber and the temperature was raised from room temperature to 250°C at 10°C/min. The mass spectrum was analyzed with a CLASS-5000 Analysis System & Software (ver. 2.23) (Shimadzu Chemical Laboratory, Tokyo).

X-ray diffraction of single crystal

The crystal sample (0.20 × 0.40 × 0.05 mm) was mounted on a glass capillary. All measurements were made with an AFC7R diffractometer (Rigaku, Tokyo, Japan) with graphite monochromated Cu-Kα radiation and a rotating anode generator. The data were collected at 23 ± 2°C using the w−2θ scan technique to a maximum 2θ value of 150.0°. Of the 5,326 reflections collected, 4,328 were unique (Rint = 0.049). The intensities of three representative reflections were measured after every 150 reflections. The structure was solved by direct methods and expanded using Fourier techniques (Beurskens et al. 1994; Sheldrick 1985). The final cycle of full-matrix least-squares refinement was based on 4,328 all unique reflections and 337 variable parameters and converged (largest parameter shift was 0.00 times its esd) with unweighted and weighted agreement factors of:

$$R = \sum ||F_o| - |F_c|| \Big/ \sum |F_o| = 0.054$$

[for 1,893 ($I > 2\sigma(I)$) cut off data]

$$R_w = \left[\sum w(F_o^2 - F_c^2)^2 \Big/ \sum w(F_o^2)^2\right]^{1/2} = 0.244$$

(for all data)

where F_o = structure factor observed; F_c = structure factor calculated; R = R factor.

The standard deviation per observation of unit weight was 0.93. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.22 and −0.22 $e^-/Å^3$, respectively. All calculations were performed using the teXsan crystallographic software package (Molecular structure, TX, USA).

Transmission electron microscopy (TEM) of microbes

Electron microscopy was carried out according to a previous report (Kobayashi et al. 2000). Each culture was filtered and the cells were collected on an HA membrane (Millipore, Germany). The membrane frozen by metal block contact was dropped into pre-cooled acetone (−80°C) containing 2%(w/v) OsO_4 and kept at the same temperature (−80°C) for 36 h in this solution. Thereafter, the solution was warmed stepwise to room temperature (−20°C, 2 h; 4°C, 2 h; room temperature, 2 h), the membrane was washed three times, and then it was infiltrated with 100% acetone. The treated membrane was embedded in epoxy resin (Epon 812). Thin sections were cut with a diamond knife on a REICHERT ULTRACUT S (Leica, Germany) ultramicrotome. After being stained with uranyl acetate and lead citrate, they were examined with a JEM−1210 electron microscope at 80 kV.

Results

Purification of violet pigment produced by *S. violacea* DSS12

S. violacea DSS12 formed violet colored colonies on modified MB agar plate (Fig. 1a). This pigment was soluble in chloroform or THF and colored blue (Fig. 1b), and showed very poor solubility in methanol, ethanol, acetone, ethyl ether, *n*-hexane and water. The crystal form of this violet pigment is needle shaped and has a metallic luster. The DSS12 strain did not produce any pigment in liquid medium. We harvested about 2.8 g

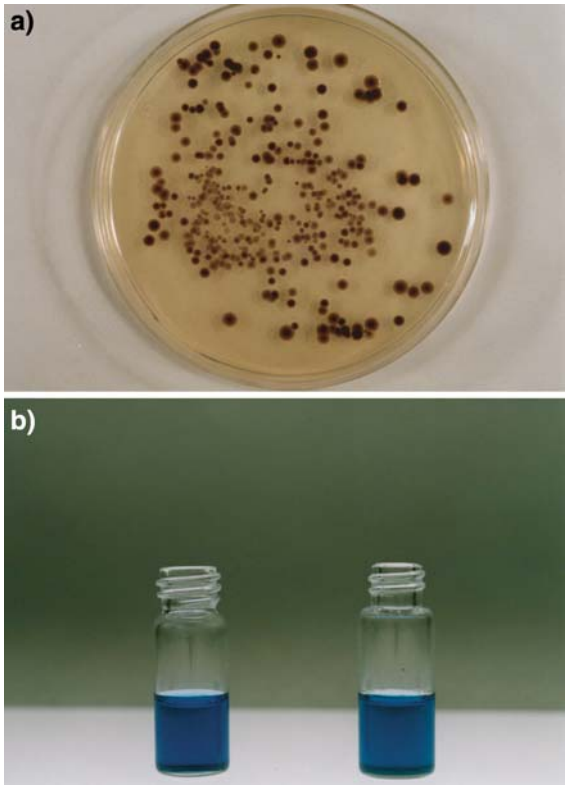

Fig. 1 *Shewanella violacea* DSS12 and its violet pigment. Colonies of *S. violacea* DSS12 grown on modified Marine broth 2216 reveal a violet pigmentation (**a**). The violet pigment is blue in THF (**b**, *left*) and chloroform (**b**, *right*)

of dry cells from 100 plates and obtained 14.6 mg of crystallized violet pigment.

The chemical structure and property of the violet pigment

The properties of the violet pigment are shown in Table 1. This pigment showed a broad absorption spectrum from 500 to 700 nm. The maximum absorbance of the violet pigment was 636 nm in chloroform and 616 nm in THF. The melting point of this violet pigment was 265°C. The molar absorption coefficient in chloroform at 636 nm was 0.64×10^3. The pigment was very stable in THF and did not fade for over 1 month. The crystal habit and system of this violet pigment were needle and triclinic, respectively, and its chemical structure was determined by X-ray diffraction of a single crystal (see supplementary data). Figure 2 shows the crystallographic character of this violet pigment. The crystal contained two THF molecules per violet pigment molecule. The pigment molecules were oriented side-by-

Table 1 Properties of violet pigment and its crystal structure

Sum formula (M. W.)	$C_{34}H_{54}N_4O_4$ (584.85)
Melting point	265°C
Absorbance maximum (nm)	636 (in chloroform)
ε 636	0.64×10^3
Crystal system	Triclinic
Lattice parameters	
A (nm)	1.0032
B (nm)	2.2395
C (nm)	0.49469
α (°)	93.537
β (°)	104.114
γ (°)	88.371s
Space group	PI (#2)
Calculated density (g cm^{-1})	1.130

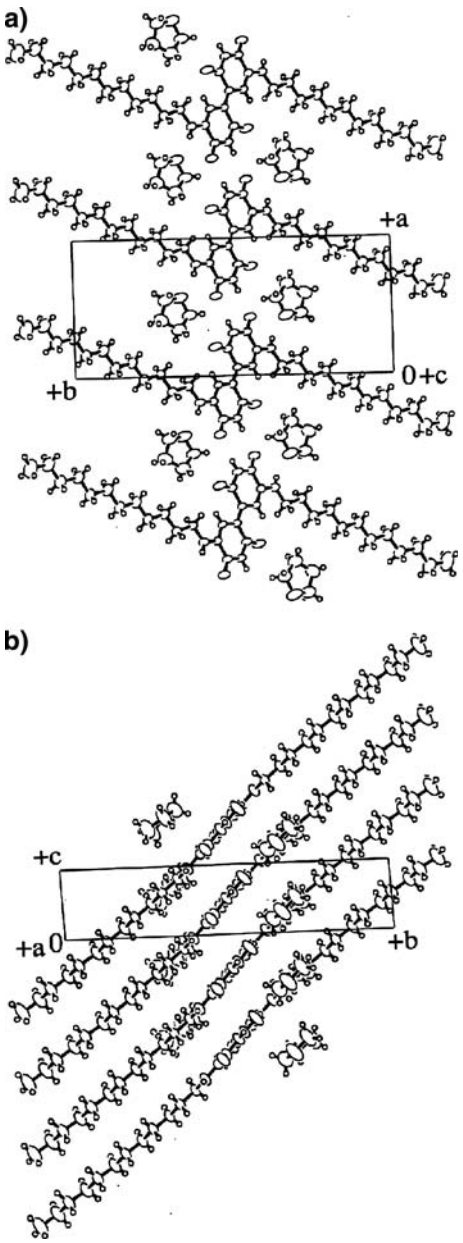

Fig. 2 Crystal projection of the violet pigment by ORTEP (Johnson 1976). **a**, **b** show the horizontal and vertical arrangements of the pigment molecules in the crystal. Lattice constants were shown as *a*, *b* and *c* in figures

side and the chromophores of these pigments are in close proximity. X-ray diffraction revealed the chemical structure of the violet pigment to be 5,5′-didodecylamino-4,4′-dihydroxy-3,3′-diazadiphenoquinone-(2,2′). The molecular weight resulting from mass spectrometry was 584 or 585 and its fragmentation pattern did not contradict its chemical structure obtained from X-ray diffraction of a single crystal (Fig. 3). This chemical structure of this new violet pigment was searched for by "STN on the web" (http://www.stnweb.cas.org/), however, no similar chemical compound was found.

TEM observation of *S. violacea* DSS12

Figure 4 shows the TEM observation of DSS12 grown on an agar plate (panel a) or in liquid medium (panel b, c). The colonies grown on an agar plate were colored violet, while liquid cultures of DSS12 were yellow. Cells grown in liquid culture had many small particles (panels b, c) while no significant material was found in cells grown on the agar plate (panel a).

Discussion

In this study, we determined the chemical structure of a new violet pigment of *S. violacea* DSS12, formed when cells were grown on solid agar medium. The chemical structure of this pigment was 5,5′-didodecylamino-4,4′-dihydroxy-3,3′-diazadiphenoquinone-(2,2′), which is an alkylated indigoidine (Fig. 5a). This violet pigment colored blue in chloroform or THF but was not soluble in other solvents including H_2O, in which it formed a violet powder. The chromophore of this violet pigment was the same as indigoidine (Fig. 5b) or close to indigo (Fig. 5c). The absorption maximum of this violet pigment in THF was 616 nm, which was about 30 nm longer than that of indigo (589 nm) or indigoidine (587 nm) (Heumann et al. 1968). Indigoidine is a dark blue pigment produced by *Erwinia chrysanthemi* or *Clavibacter michiganensis* (Heumann et al. 1968; Reverchon et al. 2002). Both solution and crystals of indigoidine or indigo were blue; however, this DSS12 pigment was violet in a crystal or solid state. This shows that the absorption maximum of this pigment shifts down from 616 to 500–580 nm. X-ray diffraction of a single crystal of this pigment showed that the pigment molecules were orientated side-by-side and that the chromophores were very close to each other (about 0.32 nm, estimated from Fig. 2b). Shimomura et al. (1995) reported that the molecules with long alkyl chains were densely packed with a π-electron overlap of chromophores, which caused a hypsochromic shift (blue shift) of the absorption spectra. The blue shift of the

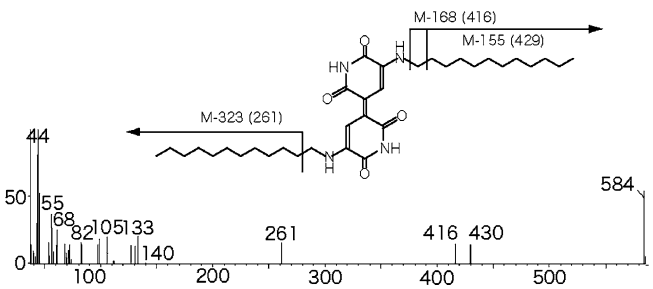

Fig. 3 Mass spectrum of the violet pigment. The chemical structure of the violet pigment proposed by X-ray diffraction of a single crystal is also indicated in this figure. The M.W. of the violet pigment is 584.85 from the result of X-ray diffraction. Major fragmentation patterns are also indicated in the chemical structure

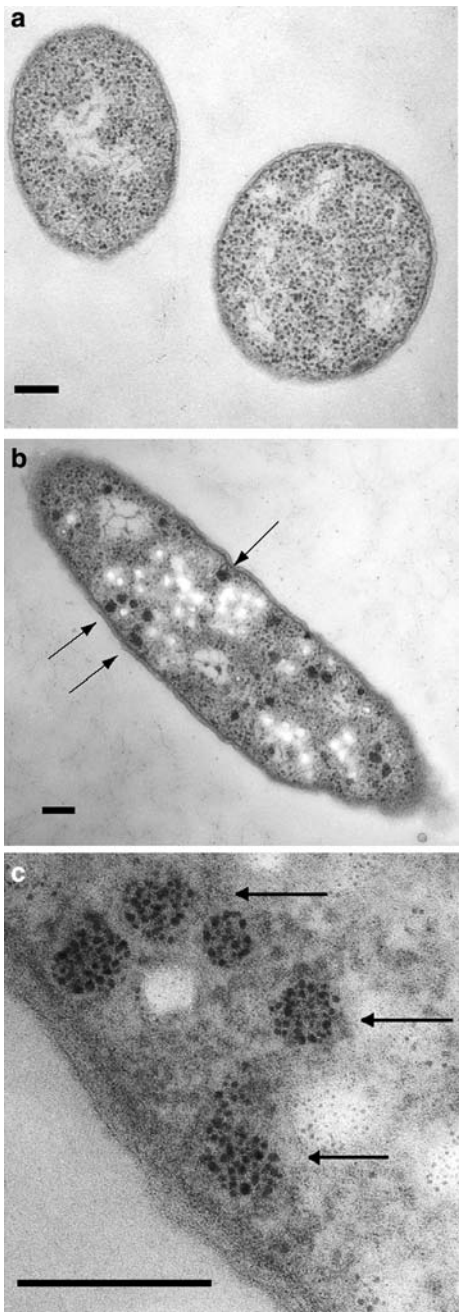

Fig. 4 Electron microscopy of *S. violacea* DSS12. DSS12 grown on the agar medium (**a**) or in liquid medium (**b**, **c**). *Arrows* indicate the structures described in the text. *Bars* indicate 200 nm

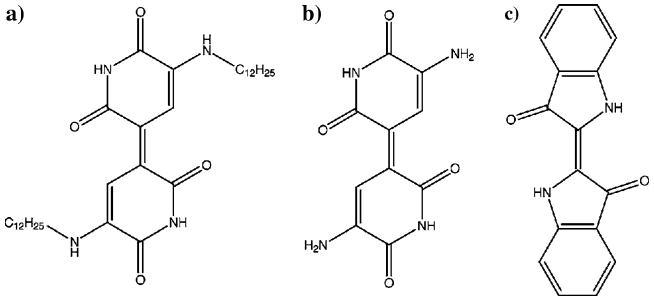

Fig. 5 The chemical structures of violet pigment (**a**) indigoidine, (**b**) and indigo (**c**)

absorption spectra causes a red shift in the observed pigment color. The violet color of *S. violacea* DSS12 was due to a blue shift of the absorption spectra of chromophores, same as in the blue pigment, indigoidine, caused by the close packing of pigment molecules.

DSS12 was previously isolated from deep-sea sediments and reported as piezophilic psychrophilic bacterium (Nogi et al. 1998). Culture conditions such as hydrostatic pressure or temperature, however, did not influence the production of violet pigment (data not shown). The TEM observation of cells showed some small structures in the cells when grown in liquid culture (Fig. 4). These structures are expected to be premature pigments such as leucoindigoidine (Heumann et al. 1968), but further studies are needed to confirm this. The violet pigment molecules may integrate into the cell membrane, due to its alkyl chain chemical structure, supporting the lack of crystal detection in cytoplasm. It was reported that indigoidine was easily oxidized and lost its color in a few days and could thus potentially operate as an oxide or radical scavenger (Reverchon et al. 2002; Giese 1986). The violet pigment of DSS12 was very stable and showed no antibiotic activity to *Escherichia coli*, but this depended on the low permeation into cells because of the crystals' insolubility in water or hydrocarbons (data not shown). The role of this new DSS12 violet pigment in the cell is still unknown, but it might be useful when applied as a dye because of its high stability and low solubility.

Recently, the sequences of *E. chrysanthemi* indigoidine synthase genes, *ind*A, *ind*B and *ind*C, were elucidated (Reverchon et al. 2002), and found in the genomes of various microorganisms such as *Thermotoga maritima*, *Sulfolobus solfataricus* and *Staphylococcus aureus* subsp. (Kuroda et al. 2001; Nelson et al. 1999; She et al. 2001). The genome project of *S. violacea* DSS12 is also in progress and is near completion. An indigoidine synthase or related genes, however, have not yet been found in the genome sequence (K. Nakasone, personal communication). The synthesis of this violet pigment might reflect a different process or pathway from that of indigoidine. Further studies will be needed to the biosynthesizes and ascertain the biological role of the 3,3′-bipyridyl pigments, including indigoidine.

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